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THE COLONISATION OF TREATED WATER
SYSTEMS BY *LEGIONELLA PNEUMOPHILA*

JULIE ROGERS

A thesis submitted in partial fulfilment of the
requirements for the Open University
for the degree of Doctor of Philosophy

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with Thames Water Utilities Plc.

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I am truly indebted to both my parents and husband whose support and encouragement have sustained my efforts.

ABSTRACT

The colonisation of treated water systems by *Legionella pneumophila*

by Julie Rogers

A two-stage continuous chemostat model of a water system was developed to investigate the ecology of *L. pneumophila*. The inoculum was derived from a calorifier responsible for an outbreak of Legionnaires' disease and contained virulent *L. pneumophila* serogroup 1 along with associated microorganisms including protozoa. The model system was supplied with filter sterilised tap water as the sole nutrient source for the growth of the mixed microbial population and was used to investigate both planktonic and attached growth.

The characteristics and reproducibility of biofilm formation on glass surfaces were examined and this provided insights into the three dimensional structure of biofilm formation. Examination of the biofilms using immunogold labelling techniques demonstrated that cells of *L. pneumophila* were present within the biofilm as microcolonies, this could indicate that the pathogen could grow extracellularly by receiving nutrient from the bacterial consortium.

The influence of plumbing material selection on the growth of *L. pneumophila* was investigated using the model system. The results obtained indicated that copper was an inhibitory material in terms of both biofouling and the growth of *L. pneumophila*. Latex and ethylene-propylene which are used for sealing plumbing systems were found to be nutrient supplying materials. These elastomeric materials supported extensive biofilms after only 24 hrs and contained high numbers of the pathogen. Steel and plastic materials supported biofilms which were intermediate in terms of total biofouling and growth of the pathogen.

The influence of water temperature on biofilm formation and the inclusion of *L. pneumophila* was also investigated. At 20 °C and 50 °C *L. pneumophila* was present in biofilms in low numbers. At 40 °C *L. pneumophila* was present in the planktonic and biofilm phases in high numbers and accounted for as much as 50 % of the biofilm flora. At 60 °C the pathogen was absent from the model system. Copper was found to be inhibitory to colonisation and growth of *L. pneumophila*.

The model system was used to develop biofilms on the surface of appropriate plumbing materials and then these were used to determine efficiency of commercially available biocides. The results demonstrated that attached bacterial populations were more resistant to biocide treatment than the planktonic phase. However, even the planktonic populations were able to survive the recommended biocide doses suggested by the manufacturers.

The implications of the data on the ecology of the pathogen is discussed in relationship to the prevention of infection. The data provides additional information on the dynamic relationship which exist within biofilms and gives additional understanding on biofilm formation.

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GLOSSARY OF TERMS

CFU colony forming units

DOT dissolved oxygen tension

IC inorganic carbon

IFA immunofluorescent assay

OC organic carbon

TOC total organic carbon

CHAPTER 1

INTRODUCTION

1.1 WHY STUDY *LEGIONELLA PNEUMOPHILA* IN WATER SYSTEMS?

Potable water systems and cooling towers are now operated under guidelines (HMSO, 1989a and 1989b) that will limit proliferation of the microorganisms, including the *Legionellaceae*. *Legionella pneumophila* can be found in a variety of water systems despite control measures and outbreaks of disease still occur.

Legionellaceae are the Gram-negative, asporogenous bacteria responsible for legionellosis. *Legionella pneumophila* is the species most commonly found to cause disease in man, although all legionella species are thought to be capable of causing pneumonia. Legionnaires' disease has a typical incubation period of 2-10 days and produces initial symptoms of malaise, myalgia, headache and chills. As the disease progresses, fever, chills and a consolidating pneumonia occurs. Death may result in some cases, particularly in immunocompromised patients. Infection by *L. pneumophila* has also resulted in outbreaks of Pontiac fever; which is an acute, febrile, non-pneumonic form of infection which is self limiting. After an incubation period of 36 hours the infection results in flu-like symptoms.

Infection by *L. pneumophila* is attributed to the inhalation of contaminated aerosols from water systems such as cooling towers, showers and nebulisers. The production of contaminated aerosols occurs because *L. pneumophila* is commonly present in a diverse range of aquatic habitats. The organism is ubiquitous in the natural environment and can be isolated from a large proportion of surface water and soil samples. It is the occurrence of *L. pneumophila* in man-made ecosystems that has led to increased human exposure and incidence of disease.

Persistence of *L. pneumophila* in water systems can be, in part, attributed to the colonisation of surfaces by aquatic bacteria (in biofilm) and the inclusion of *L.*

pneumophila into this biofilm. Biofilms pose additional problems for the control of pathogens since biocide penetration may be limited and systems may contain undetected pathogens on surfaces. The intracellular growth of *L. pneumophila* within aquatic protozoa not only increases bacterial numbers, but also make control treatments less effective since these hosts may provide protection from some of the biocides used.

Further ecological studies of *L. pneumophila* are required to aid the understanding of those factors which lead to the proliferation of the organism within water supplies and to determine suitable control measures. Studies within water systems or cooling towers have inherent problems of control, reproducibility and statistical testing. A model system would be more suitable for the determination of more precise information regarding the environmental factors which influence legionella numbers within aquatic systems.

1.2 AEROSOL AND HUMAN INFECTION

Legionella pneumophila was classified as a new bacterium in the family *Legionellaceae* in 1979 by Brenner *et al.* There are a further 39 additional species that have been identified as members of this genus (Benson *et al.* 1991 & 1989, Bercovier *et al.* 1986, Bornstein *et al.* 1989, Dennis *et al.* 1993, Thacker *et al.* 1988, 1989, 1991, & 1992, Verma *et al.* 1992 and Wilkinson *et al.* 1987 & 1988) and the list is still growing. Speciation of the members of the *Legionellaceae* may be carried out using biochemical tests, gas-liquid chromatography or isoprenoid quinone profiles. To achieve a definitive identification the use of DNA hybridization studies is recommended, but since these techniques are not routinely available presumptive identification is achieved using serological methods (Harrison and Taylor, 1988).

Legionellae are divided into subgroups by their reaction with hyperimmune rabbit

antisera containing antibodies to lipopolysaccharide or "o" antigens. The method uses a simple indirect immunofluorescent test (IFA) which provides rapid and fairly reliable results for most species. For *L. pneumophila* in particular, there is little cross reactivity with other serogroups. Since *L. pneumophila* serogroup 1 is most often the cause of disease and appears to be the most common species in man-made ecosystems, the serological methods used can be considered adequate for most isolates that would be routinely cultured.

Legionella pneumophila has 14 serogroups, of which serogroup 1 is most prevalent. In their typing system Watkins *et al.* (1985) divided serogroup 1 into 3 major subgroups known as Pontiac, OLDA and Bellingham. Of these subgroups Pontiac is the most common clinical isolate but is less readily isolated from environmental samples than the other two subtypes. The North American panel of monoclonal antibodies (Joly *et al.*, 1983) classifies several groups of *L. pneumophila* serogroup 1 which fall within the British Pontiac group, several groups which fall within OLDA subgrouping and a group which is equivalent to subgroup Bellingham.

Of the other species identified, at least 16 species have been responsible for outbreaks of legionellosis (Fallon & Rowbotham 1990, Fields *et al.* 1990, Herwaldt *et al.* 1984 Joly *et al.* 1986 and Lee & West 1991). Other species have not yet been implicated as pathogens (Dennis *et al.*, 1993).

Early epidemiological data provided circumstantial evidence for the aerosol route of infection of *L. pneumophila* (Thacker *et al.*, 1978, Dondero *et al.*, 1980 and Bleckmon *et al.*, 1981). Direct evidence for the aerosol route of infection in guinea pigs and Rhesus monkeys was provided by Baskerville *et al.* (1981). Not only were infection and proliferation of the organisms achieved, but guinea pigs displayed symptoms and histopathology similar to that observed in man and thus provided a suitable animal model. Horwitz and Silverstein (1980) were able to demonstrate that growth of *L.*

pneumophila occurred in the macrophages of the lung. As *L. pneumophila* did not grow extracellularly or in leukocytes, it was concluded that the bacterium was a facultative intracellular parasite in the host.

The demonstration of the aerosol route of infection led investigators of outbreaks to pinpoint particular water systems responsible for their generation. Aerosolisation of tap water from jet nebulisers and a portable room humidifier were found to be responsible for five cases of legionellosis in patients in the University of Chicago Hospital (Arnow *et al.*, 1982). Although the same strain was isolated from both hot and cold water supplies, comparison with uninfected patients showed the significant factor to be the use of respiratory equipment for at least two days of the incubation period.

However, the presence of aerosols containing *L. pneumophila* was not necessarily linked to disease. In a routine examination of water systems in a large building, *L. pneumophila* was isolated from a shower hot water supply (Dennis *et al.*, 1984). Water samples contained 10^3 culturable legionellae per liter. Whilst the shower was running viable *L. pneumophila* were recovered from air samples at a concentration of 2 or 3 colonies of *L. pneumophila* in 7500 l of air. In an effort to establish the effect of the contamination on human health 90 blood samples were obtained from staff. There was no evidence of clinical illness but two members of staff were found to have titers $>1/32$ against heat killed antigen prepared against this strain, although neither of these staff had used the shower. A similar, non-outbreak situation was examined by Oppenheim *et al.* (1987) who found contamination by *L. pneumophila* serogroup 1 and 4 in dental station outlets of the London Hospital Dental Institute. Members of staff and patients were screened using legionellae antibodies, since contaminated aerosol generation was considered probable, but doses were too low to cause infection or stimulate immune responses.

Despite the demonstrated absence of infection following aerosolisation of *L. pneumophila* in some studies, the aerosol route for infection has now been well established and cases continue to be reported. Addiss *et al.* (1989) supplied evidence for cooling tower dissemination for a community outbreak with patients infected over a distance of 1.6 - 3.2 km away from the tower, which suggests that *L. pneumophila* could survive within aerosols over long distances, if the humidity was high and atmospheric temperature inversions occurred.

Investigations involving the factors that permitted bacterial survival within aerosols had been ongoing since 1980 when Berendt investigated the effect of relative humidity on the survival of *L. pneumophila*. Aerosols of the bacterium, suspended in tryptose-saline solution were disseminated into a humidity controlled sphere. At 80 % relative humidity the bacterial aerosols were stable with a half life of 15.6 min. At 50 % the half life was 10.3 min and at 30 % the bacterium was less stable with a half life of 3.2 min. Further work by Berendt (1981) showed that *L. pneumophila* was more stable in aerosols when a more natural suspending fluid, a cyanobacterial extract from *Fischerella* sp., was used. If *L. pneumophila* was disseminated from distilled water the organism was found to be unstable and no viable bacteria could be recovered after 18 min.

The influence of metabolic status and relative humidity on the survival of *L. pneumophila* in aerosols was examined by Hambleton *et al.* (1983 and 1984). A rotating drum was used to store aerosols of *L. pneumophila* with a stable tracer (*Bacillus subtilis*). The authors found *L. pneumophila* survived best at 65 % relative humidity, less well 90 % relative humidity and poorly at 30 % relative humidity. Furthermore, stationary phase broth-grown organisms survived better in the aerosol than exponentially growing bacteria.

The sites of isolation of the strain of *L. pneumophila* were demonstrated to have an

impact on the ability of the organism to survive in aerosols of 20 and 60 % relative humidity by Dennis *et al* (1988) using 14 strains of *L. pneumophila*. Strains from patients or from environments associated with infection were found to survive better than strains from environmental sites unassociated with infection with *L. bozmannii* and *L. micdadei* surviving better than *L. pneumophila* under the same environmental conditions.

1.3 THE DISTRIBUTION OF LEGIONELLAE IN NATURAL HABITATS

Legionella pneumophila was isolated from soil and river water samples during an investigation of an outbreak of Legionnaires' disease (Politi *et al.*, 1979). Although a cooling tower also contained *L. pneumophila*, the failure of water treatment to prevent further infection indicated that the environment may have been responsible for infection. Further sampling of the local area demonstrated the common occurrence of *L. pneumophila* in the natural environment with 30 % of samples containing the organism (Morris *et al.*, 1979).

The discovery of *L. pneumophila* in natural conditions led to the investigation of natural habitats (Fliermans *et al.*, 1979). Over 90 lake and pond waters contained 10^5 to 10^6 direct fluorescent-antibody (DFA) positive cells of *L. pneumophila*. It was concluded that *L. pneumophila* existed as part of the natural microbial community of the aquatic ecosystem as a small proportion (approximately 1%) of the total flora. The ubiquitous nature of the organism was attributed to the tolerance of elevated temperatures, low pH and low oxygen concentrations (Fliermans *et al.*, 1981). Although the pathogen seemed ubiquitous it was not necessarily present at all sampling sites, for example, *L. pneumophila* could not be detected in chemically contaminated ground water despite a wide diversity of other contaminating bacteria (Spino *et al.*, 1984). In addition, the first polyclonal antibodies that were developed and used during this time had questionable

specificity.

The niche occupied by the *Legionellaceae* was not confined to freshwater habitats. *Legionella bozmannii*, *L. micdadei*, *L. bozmannii*, *L. dumoffii*, *L. gormanii* and *L. pneumophila* being isolated at densities of 10^4 immunolabelled cells ml^{-1} , in freshwater, estuarine water and marine waters (Ortiz-Roque & Hazen, 1987). *L. pneumophila*, *L. micdadei* and *L. gormanii* could also be found in rainwater which could possibly explain the occurrence of legionellae in the bracts of epiphytic plants 30 ft above ground level (Turner *et al.* 1984). In several investigations the detection of legionellae in water samples led to an investigation of pneumonia cases and legionellosis was retrospectively diagnosed (Ortiz-Roque & Hazen, 1987). The detection and persistence of *L. longbeachae* in soil and potting composts was also responsible for sporadic cases of legionellosis (Steele *et al.*, 1990).

Hot spring spa water containing 15 species of *Legionellaceae* caused legionellosis but the number of cases and the severity of disease was low (Bornstein *et al.*, 1989). Other heated waters have also been associated with infection with *Legionellaceae*. Following the eruption of Mt. St. Helens in 1980, the lake habitat was converted to a hypereutrophic environment due to nutrient enrichment with ash and organic matter (Tison *et al.*, 1983). After research workers suffered flu-like symptoms virulent *L. pneumophila*, *L. micdadei*, *L. gormanii*, *L. dumoffii* and *L. bozmannii* were isolated from the blast zone, but no cases of legionellosis were confirmed. The high density of the pathogen was attributed to elevated temperatures (40-60 °C) and elevated carbon content of the water (250mg/l). A new species, *L. sainthelensis*, was also identified from spring water effected by the eruptions (Cambell *et al.*, 1984). Elevated temperatures were also correlated with incidence of legionella in natural waters from hydrothermal areas of Portugal (Verissimo *et al.*, 1991).

1.4 THE DISTRIBUTION OF LEGIONELLAE IN MAN-MADE ECOSYSTEMS

1.4.1 Cooling towers

The first cases of Pontiac fever were described by Glick *et al.* (1978). There were at least 144 cases of infection, including 95 members of staff at the health department building in Pontiac. The high attack rate and rapid onset of disease (after 36 h) led to an epidemiological investigation, and spread of contaminated aerosol was found to be due to the close proximity of intake and exit air of the air-conditioning system.

A hospital associated outbreak of Legionnaires' disease coincided with the temporary use of an auxiliary air-conditioning system. The detection of *L. pneumophila* in the untreated cooling tower water, combined with evidence of aerosol drift, provided the first evidence of airborne transmission of infection (Dondero *et al.*, 1980). Similarly, partial shutdown of facilities in an automobile factory resulted in 317 cases of Pontiac fever, *L. feeleii* was isolated from coolant water and smoke tracers demonstrated aerosol drift (Herwaldt *et al.*, 1984). The drift of air-conditioning vapour over a golf course also resulted in infection and investigations showed that *L. pneumophila* was present in the air-conditioning system at a concentration of 10^5 cells ml⁻¹ (Cordes *et al.*, (1980).

The reports of outbreaks of Legionnaires' disease led several investigations to determine the extent of colonisation of water systems by *L. pneumophila*. Around 12 % of all wet-type heat rejection cooling towers in Vermont (USA) were found to contain *L. pneumophila* (Witherell *et al.*, 1984). In Paris, 80% of air-conditioning systems, 83% of cooling towers and 16% of air wash systems sampled were found to be contaminated by legionellae (Desrosiers *et al.*, 1984). The diversity of contamination was indicated when *L. bozemanii*, *L. dumoffii*, *L. micdadei*, *L. gormanii* and *L. pneumophila* were found at concentrations of up to 10^5 cfu ml⁻¹ in another study of air

conditioning cooling towers (Negron-Alvira *et al.*, 1988). Widespread contamination of large power plant cooling towers was also demonstrated when 98% of all samples contained legionellae (Solomon *et al.*, 1984). The species isolated were *L. oakridgensis*, *L. gormanii*, *L. bozemanii* and most abundantly *L. pneumophila*, with a seasonal peak of densities being evident in spring (Tyndall *et al.*, 1984). Intraperitoneal injection of water samples into guinea pigs confirmed that 51 of the 143 samples were infective (Christensen *et al.*, 1984).

The occurrence of legionellae in cooling towers despite biocide treatment resulted in several investigations to determine biocide suitability. *L. oakridgensis* was found in towers treated with a twice daily dose of sodium hyperchlorite to give a residual of 0.7 mg/l chlorine (Tyndall *et al.*, 1983). Thiocarbamate-based biocides also appeared ineffective since *L. pneumophila* could be consistently isolated from treated towers (Grow *et al.*, 1984). Following an outbreak of Pontiac fever a determined effort was made to eradicate *L. pneumophila* from an office building (Friedman *et al.*, 1987). The cooling tower was treated with 10 mg/l free chlorine for 12h and then a residual of 2 mg/l was maintained. As water samples still contained *L. pneumophila*, hyperchlorination with 10 mg/l chlorine for 60 h, followed by maintenance of a 2 mg/l residual chlorine was necessary to control the *L. pneumophila* in the tower.

As there were several cases of hospital acquired legionellosis reported, this caused great public concern over *L. pneumophila* in cooling towers (Fischer-Hoch *et al.* 1981, O'Mahoney *et al.* 1990 and Mitchell *et al.* 1990). There were 68 confirmed cases, with 22 deaths, due to infection with *L. pneumophila* in Stafford hospital outpatients department (O'Mahoney *et al.*, 1990). Several factors were implicated in aiding the spread of infection : 1) the air-conditioning chiller was horizontal to the cooling tower drain so waste water could flow into the unit. 2) Debris from building work partially blocked the drain and a fault on the conductivity control valve resulted in continual loss of water from the cooling tower. Some of the water was observed to bubble out at the

chiller unit and the epidemic strain was isolated from the mastic material surrounding the unit. 3) Aerosols were shown to be produced from the original equipment in laboratory trials. Poor cooling tower maintenance (Mitchell *et al.*, 1990) and failure of disinfection treatments (Fischer-Hoch *et al.* 1981) provided the opportunity for *L. pneumophila* to grow within water systems.

1.4.2 Drinking water supply system

The common occurrence of *L. pneumophila* in water systems suggested that they could enter with supply water. However, in several studies there was no evidence for this, for example, *L. pneumophila* was commonly isolated from plumbing systems of hospitals and hotels but were not detected in mains water (Tobin *et al.*, 1981a, b). Raw waters were found to contain $10^4 - 10^5$ *L. pneumophila* ml⁻¹ but after treatment improved water quality resulted in a two fold reduction in the numbers of *L. pneumophila* using DFA and no *L. pneumophila* could be cultured (Colbourne & Trew 1986, States *et al.* 1983 and Tison & Seidler 1983). Eventually *L. pneumophila* was cultured from treated waters, however, only on one occasion despite 40% of samples containing *L. pneumophila* as indicated by DFA (Colbourne *et al.*, 1988a). The data suggested that *L. pneumophila* occurred in source waters sporadically and in very low numbers. Those *L. pneumophila* gaining access to the distribution system could grow under more favourable conditions until numbers were detectable.

Further work on London's water supply suggested that aquifers could also be a habitat for *L. pneumophila* and demonstrated that the presence of the organism in source water was linked to occurrence in domestic supply (Colbourne & Dennis, 1989). Since up to 60% of buildings in London were supplied with water containing *L. pneumophila* with a seasonal peak in warmer months, it was concluded that methods of water treatment were insufficient to eradicate the pathogen (Colbourne *et al.* 1988).

In an attempt to conclusively link water supply to occurrence of *L. pneumophila* in hospital waters, seven hospitals with the same water supply were sampled. *Legionella pneumophila* was not detectable in raw waters, nor in two of the hospital waters but could be isolated in some samples from the other hospitals. The distribution of monoclonal subtypes and plasmid types led to the conclusion that the diverse population of *L. pneumophila* was due to the unique ecosystem within each building (Bezanson *et al.*, 1992)

1.4.3 Cold potable water

Legionella pneumophila has been shown to occur in water storage and distribution systems of hospitals and hotels and cause infection (Tobin *et al.*, 1981a,b; Wadowsky *et al.*, 1982). Although the transient occurrence of the pathogen within the water systems can result in failure to detect the organism from systems previously known to contain *L. pneumophila* (States *et al.*, 1987). Surveys in Paris and London have shown that 24-30% of cold water samples and 9-21% of cold water storage tank samples may contain *L. pneumophila* (Dennis *et al.* (1982) & Desplaces *et al.* (1984).

Legionella pneumophila were detected in cold tap waters at a concentration of 10^2 - 10^4 cells l^{-1} in only 3% of buildings surveyed by Colbourne & Trew (1984). Despite the low incidence and density of the pathogen in cold water samples reported in the literature cases of infection due to cold water contamination have nevertheless occurred. Nosocomial infection has been attributed to inhalation of aerosolised cold water (Ruf *et al.*, 1988) and washing of sternal wounds with contaminated water following surgery (Lowry *et al.*, 1991).

1.4.4 Hot water systems

Nosocomial outbreaks of Legionnaires' disease have been linked to contamination of hospital hot water supplies, with *L. pneumophila* being isolated from hot water storage units (Tobin *et al.*, 1981) and tap water (Fischer-Hoch *et al.*, 1981). The widespread occurrence of *L. pneumophila* in one hospital resulted in 50 cases of Legionnaires' disease and the isolation of the organism from showers, taps, a bed pan flusher and water storage tanks (Stout *et al.*, 1982). *Legionella micdadei* has also been isolated from hospital hot water systems (Brown *et al.*, 1983).

An epidemiological link was demonstrated between hot water supply and legionella infection during a study where ten hospitals were sampled for *L. pneumophila* and *L. micdadei*. The elimination of further cases of legionellosis was achieved by maintaining water temperatures above 54 °C after heat flushing at 60-77 °C for three days (Best *et al.*, 1983). The failure of operating hot water systems at sufficiently high temperature to prevent growth of *L. pneumophila* has resulted in cases of legionellosis. Hot water systems operating in a temperature range of 35-69 °C were found to contain legionella in 85% of hot water sample in one hospital where there had been 35 cases of Legionnaires' disease. The combined effect of low operating temperatures (31 °C) and stagnation in one hospital allowed *L. pneumophila* to persist in hot water tanks for over a year suggesting that warm stagnant water encouraged growth of *L. pneumophila* (Ciesielski *et al.*, 1984).

Legionella pneumophila, *L. erythra*, *L. jordanis* and *L. rubrilucens* were isolated from a hospital hot water system which was operating at 35 °C and had 13 fatal cases of Legionnaires' disease. Contaminated tap water had been used to fill respiratory devices which had contributed to infection (Ruggeinin *et al.*, 1989).

Infection by *L. pneumophila* occurred after a pressure failure in hospital water supply

resulting in dirty-brown water. *L. pneumophila* was isolated from 30% of shower outlets prior to an experimental pressure drop, when *L. pneumophila* was then found in 50% of shower outlets and 80% of water samples. Water samples became cloudy, as was observed during the initial pressure drop, suggesting that the elevated numbers of *L. pneumophila* could possibly due to the loss of biofilm bacteria from pipe surfaces (Shands *et al.*, 1985). The water supply was treated to a concentration of 2 mg/ml which resulted in a reduction, but not elimination of *L. pneumophila*. Further evidence for the control of *L. pneumophila* by use of chlorine was provided when a hospital using unchlorinated water was sampled, 15% of outlets were found to contain *L. pneumophila* and infection with the pathogen accounted for 30% of all pneumonias. A comparative hospital having chlorine treated waters was found to contain no detectable *L. pneumophila* (Johnson *et al.*, 1985).

Hospitals were not the only large buildings to be associated with legionella infection, one particular hotel in the Virgin Islands had been the source of 27 cases of Legionnaires' disease over a three year period. Hot and cold waters were found to contain *L. pneumophila* and this was attributed to the potable water (which was supplied from a combination of rainwater, city supply water and private well water) and the storage cistern which contained 50-70 cfu ml⁻¹ (Schlech *et al.*, 1985). Even when infection by *L. pneumophila* had not been reported, hotels were often found to be contaminated. In one study 32% of plumbing systems samples, 17% of hot waters and 17% of shower swabs were found to contain *L. pneumophila* (Dennis *et al.*, 1982)

Hot water samples, hot water tanks and taps were found to contain *L. pneumophila* in domestic premises (Desplaces *et al.*, 1984). Several authors provided evidence that electric storage water heaters were able to encourage the growth of *L. pneumophila* since their design produced a cooler zone of water at the bottom of the tank. Gas heaters which are heated from below were found to operate at temperatures too hot to permit growth of the pathogen (Alary & Joly 1991, Lee *et al.* 1988 and Witherell *et al.*

1988).

1.4.5 Legionellae in biofilms

A biofilm consists of cells immobilised at an interface or surface (Marshall, 1976). These cells are often embedded in an organic polymer matrix produced by the microorganisms (Ellwood *et al.*, 1982 and Costerton *et al.* 1987). However, a significant portion of the biofilm may be inorganic, since abiotic substances are held within the biotic matrix (Characklis and Marshall, 1989).

Biofilms are ubiquitous in the natural environment, for example, their presence has been demonstrated on riverbed stones (Geesey *et al.*, 1978), in industrial water systems (Mittel and Geesey, 1987), in drinking water pipelines (LeChevallier *et al.*, 1987), on mucosal surfaces (Freter *et al.*, 1981), and on teeth (Gibbons and van Houte, 1975). For more detailed review the author refers readers to Characklis and Marshall (1989), Costerton *et al.* (1987), Denyer *et al.* (1993), Ellwood *et al.*, (1982), Marshall (1989), Melo *et al.* (1992), Lappin-Scott and Costerton (1989) and Loosedrecht *et al.* (1990).

The presence of biofilms may be beneficial to mankind. For example, the presence of commensal flora on the skin and mucosal surfaces provides protection from the ingress of pathogenic species (Cheng *et al.*, 1981), the presence of degrading populations of microorganisms on the gut walls aid digestion and absorption of nutrients (Cheng and Costerton, 1980) and the presence of bacterial populations on water treatment plant surfaces is used to improve water quality.

However, the presence of biofilms may have effects which are potentially harmful or result in the unwelcome degradation of the surfaces. The formation of dental plaque on teeth results in the entrapment of extracellular byproducts of sucrose utilisation leading to the degradation of tooth enamel (Sonnenwirth *et al.* 1980). The use of implants in

medicine includes joint and vascular prostheses, urinary catheters, intravascular catheters and endotracheal tubes. The presence of biofilms on these surfaces may lead to infections (Phillips, 1989) including septicemia, peritonitis, endocarditis, mediastinitis, meningitis and lead to the loosening of joint prostheses (Dickinson and Bisno, 1989).

There may also be additional economic costs if biofilms are present in industrial applications since their presence may lead to loss of heat transfer, loss of fluid flow or corrosion (Lappin-Scott and Costerton, 1989). For example, metal corrosion, souring of oil and the plugging of oil reserves are a consequence of growth of biofilms containing sulphate reducing bacteria in the North Sea oil recovery industry (Hamilton, 1983). The colonisation of ship hulls leads to reduced speed, increased fuel costs and requires ships to be removed to dry docks for cleaning (Dempsey, 1981).

Water systems contain bacteria that are able to survive and grow despite the low nutrient status of their surrounding environment. The growth and survival of microorganisms, along with macroorganisms, in the drinking water systems has been recognised for many years (Whipple *et al.*, 1927). Victoreen (1969) suggested that the mixed population of microorganisms occurring on the tube/water interface constituted a specialised ecosystem. The microorganisms are able to gain sufficient nutrients by developing within the biofilm at the interface between the water and the plumbing material surface. The biofilm is composed of a diverse range of microorganisms (including bacteria, fungi, and protozoa and possibly viruses) along with their extracellular products. The material supporting the biofilm may also become incorporated into the biofilm, for example, metal corrosion products and the water surrounding the biofilm which contained diffusible and absorbable constituents such as humic acids, oxygen and inorganic salts (Keevil *et al.*, 1990).

There were several investigations where *L. pneumophila* was found within biofilms

attached to the inside of plumbing fixtures. Following six cases of Legionnaires' disease in a Chicago hospital *L. pneumophila* was cultured from the internal surfaces of shower heads (Cordes *et al.*, 1981). Sterilisation of the shower head with ethylene dioxide provided only short term eradication of the organism. Samples of biofilm provided the only detectable *L. pneumophila* during some investigations (Brown *et al.*, 1982), suggesting that *L. pneumophila* could be concentrated within biofilms.

In a survey of hospitals, institutions and homes *L. pneumophila* and *L. micdadei* were isolated from tap, shower and valve surfaces. The sediment from hot water tanks operating at 32-42°C contained high numbers of *L. pneumophila* but these were absent from heaters operating at 71°C, indicating that heat could be used to eradicate *L. pneumophila* from biofilms (Wadowsky *et al.*, 1982).

Following an outbreak of Legionnaires' disease at Kingston hospital cold water systems were treated with 2mg/l chlorine and hot water temperatures were raised to 55 °C. The periodic recovery of *L. pneumophila* indicated that control measures had been unsuccessful in eradicating the organism. *L. pneumophila* was recovered from 31% of samples of rubber materials from taps and showers and only when these components were replaced did the system become free of contamination (Colbourne *et al.* (1984) & Fischer-Hoch *et al.* (1984)).

1.4.6 Contamination of other man-made water environments

Devices supplied with contaminated tap water were also responsible for cases of infection. Contaminated distilled water containing *L. dumoffii* led to five cases of Legionnaires' disease when it was used in respiratory therapy equipment (Joly *et al.*, 1986). Nebuliser water was also shown to contain legionella and incorrect fitting of the equipment was demonstrated to lead to aerosolisation of the bacterium (Gorman *et al.*, 1980). *Legionella pneumophila* and *Pseudomonas aeruginosa* were isolated from

humidification trays in a neonatal unit due to contamination of the supply water, which was operating at 45 °C (Verrissimo *et al.*, 1990). Another possible source of infection in the health-care environment was demonstrated after *L. pneumophila*, *Pseudomonas aeruginosa* and amoebae capable of supporting the growth of *L. pneumophila* were found in eyewash station waters (Pasko-Kolva *et al.*, 1991).

A variety of recreational waters were found to contain *L. pneumophila*, 12% of swimming pool waters, 28% of whirlpool samples and 9% of polluted surface waters were found to contain the pathogen in one study (Seidel *et al.*, 1986). There was an outbreak of Pontiac fever due to infection with *L. pneumophila* associated with the use of treated whirlpools (Witherell *et al.*, 1983). It has been demonstrated that the duration of whirlpool use and lack of ventilation were factors implicated in infection (Spitalny *et al.*, 1983). Whirlpools containing contaminated water were found to produce aerosols with droplets of a sufficient size to contain *L. pneumophila* but small enough to enter the tracheobronchial tree (Mangione *et al.*, 1985). Isolation of a different species, *L. micdadei* from a whirlpool spa in a leisure complex was achieved following one outbreak (Fallon *et al.*, 1990). The outbreak was responsible for acute illness in 96% of 178 visitors who had visited the complex over a four day period in New Year (Goldberg *et al.*, 1989).

1.5 GROWTH OF *L. PNEUMOPHILA* IN MODEL ECOSYSTEMS

1.5.1 Growth in sterile water

The longevity of legionella in sterile distilled water; sterile tap water and treated cooling tower water has been compared (Skaliy & McEachern, 1979). No growth occurred and legionellae numbers gradually declined. When biocide was present no bacteria could be cultured. In non-treated waters some of the *Legionella* spp. survived for over one year but the numbers were less and in deionised water the organism could not be detected

after several weeks.

Studies of the survival of a strain *L. pneumophila* (serogroup 1 Pontiac) in a range of sterile waters showed that after 65 days all waters assessed contained viable legionella but numbers had been reduced from 1000 to $<500 \text{ cfu ml}^{-1}$ (West *et al.*, 1990). In chemostat culture at a dilution rate of 0.03 h^{-1} and using sterile water as the nutrient, the same strain of *L. pneumophila* was found to wash out of pure culture. The data indicated that *L. pneumophila* could survive but not grow in sterile water, and that viability was gradually reduced.

1.5.2 Planktonic growth under non-sterile conditions

Successful growth of *L. pneumophila* in water was eventually achieved by using a naturally occurring microbial population from a shower head and a hot water tank, this suggested that the other microorganisms were supporting the growth of the pathogen (Yee & Wadowsky, 1982). Multiplication of the *L. pneumophila* occurred at 25, 32 and 37 °C, with a doubling time of 28.8h at 37°C. At 42 and 45 °C the numbers of *L. pneumophila* were undetectable after 14 and 28 days respectively (Wadowsky *et al.*, 1985). Using the serial transfer of the mixed population of microorganism into buffers, *L. pneumophila* was found to grow at pH 5.5-9.2. This was a wider pH range than that indicated by isolation from water systems which operated at pH 7.1-8.2. The pathogen was able to grow in oxygen levels of 6.0-6.7 mg/l, but under anaerobic conditions (less than 22 mg/l oxygen) the numbers of *L. pneumophila* declined over a 28 day period.

In unsterile water *L. pneumophila* could be maintained, but as in previous work in sterile water, numbers declined to below detection after 14 days when no associated microorganisms were present (Stout *et al.*, 1985). By increasing the amount of sediment present in the water, growth of *L. pneumophila* could be achieved, indicating that attachment of the microorganisms encouraged multiplication. Satellite growth of *L.*

pneumophila on cysteine deficient media indicated that *Flavobacterium*, *Pseudomonas*, *Alcaligenes* and *Acinetobacter* sp. were supplementing the growth of the pathogen.

An alternative approach to the use of the whole microbial community for modelling the growth of *L. pneumophila* used a combination of a small number of specific organisms isolated from the environment. The combination of *L. pneumophila*, *Pseudomonas paucimobilis* and the amoeba *Hartmanella vermiformis* was successful in achieving growth of *L. pneumophila* by intracellular multiplication in the amoeba (Wadowsky *et al.*, 1991). Growth of *L. pneumophila* did not occur in the presence of *Pseudomonas paucimobilis* alone or when amoeba were from non-environmental sources.

1.5.3 Attached growth under non-sterile conditions

The influence of biofilms on the survival of *L. pneumophila* was tested using a variety of plumbing materials (Colbourne *et al.*, 1984). Materials were immersed into unsterile water containing *L. pneumophila* and incubated at 30 °C for six weeks with the water being replaced twice weekly. In water in contact with control glass surfaces the numbers of *L. pneumophila* declined over the test period and were undetectable after twelve weeks. Water in contact with the paraffin wax positive control and rubber plumbing materials were found to contain *L. pneumophila* for the duration of the test indicating that growth had occurred. The growth was also evident by the occurrence of biofilm on the surface of the materials.

A recirculating model of a water system using a mixed population inoculated with *L. pneumophila* demonstrated that stainless steel, aluminium, silicone, rubber and glass could support biofilms which contained *L. pneumophila* (Schofield and Wright, 1984). Rubber had the greatest number of *L. pneumophila* in the biofilm and silicone > stainless steel > glass. Copper was not colonised. Since *L. pneumophila* was observed in microcolonies in almost pure cultures on the surfaces and few amoebae could be

observed, growth of *L. pneumophila* was attributed to nutrient supply by other biofilm bacteria. The legionella could be isolated from the materials after 14 months in storage, indicating that *L. pneumophila* could survive for long periods of time in unsterile, stagnant water (Schofield, 1985).

Samples of stainless steel, copper, aluminium, latex and synthetic rubbers were incubated in a variety of sterile and non-sterile waters. Legionella were undetectable after two weeks in all waters with the exception of unsterile hot tap water. Biofilms could be observed on all surfaces but copper was poorly colonised. *Legionella pneumophila* were observed as individual cells and in small groups on the surface of the rubber materials (Schofield and Locci, 1985).

1.6 GROWTH OF *LEGIONELLA PNEUMOPHILA* WITH OTHER MICROORGANISMS

1.6.1 Growth with protozoa

Growth with amoebae

The amoebae, *Acanthamoeba castellanii* and *Acanthamoeba polyphaga* were demonstrated to be hosts for the intracellular replication of *L. pneumophila*. These amoebae, which can commonly be isolated from soil and humidifiers, were found to be so heavily infected that they lysed (Rowbotham, 1980). Mature cysts were resistant to infection and no legionella could be detected in the flagellate phase of *Naegleria gruberi* and *Naegleria jardini*.

Several other species of amoebae were demonstrated as potential hosts for *L. pneumophila* including *Naegleria lovaniensis*, *Acanthamoeba royreba*, (Tyndall &

Dominique, 1982) *Acanthamoeba palestinensis*, (Skinner *et al.*, 1983) and *Naegleria fowleri* (Newsome *et al.*, 1985). In all cases, growth of *L. pneumophila* was intracellular and diffusible extracellular products produced by amoebae were not capable of supporting growth of the *L. pneumophila* (Holden *et al.*, 1984a,b). However, the relationship between parasitic bacteria and their amoebal host was not simple, cohabitation or the reductions in the numbers of *L. pneumophila* indicated that selection pressures were occurring (Tyndall & Dominique, 1982). Another factor that influenced results was the type of growth media used, by varying the suspending fluids the infectivity of *L. pneumophila* could be either induced or prevented (Newsome *et al.*, 1985).

Growth with other eukaryotes

Amoebae were not the only eukaryotes capable of acting as hosts to *L. pneumophila*. Intracellular multiplication in the ciliated holotrich *Tetraymena pyriformis* has been demonstrated using tap water as the nutrient source (Fields *et al.*, 1984). Intracellular growth of *L. pneumophila* was also observed in a cooling tower derived strain of *Tertrahymena* sp. The protozoa was found to contain 1-30 bacterial cells and it was suggested that protection from cold conditions could be conferred by the intracellular location of *L. pneumophila* (Barbaree *et al.*, 1986). Comparison of retention times in the ciliate of *L. pneumophila* and *Escherichia coli* showed prolonged survival of *L. pneumophila* within vacuoles on the ciliate (Smith-Somerville, *et al.*, 1986).

One benefit to researchers of the intracellular growth of *L. pneumophila* within protozoa was that it provided an alternative mechanism for the enrichment of *L. pneumophila* from clinical samples (Rowbotham *et al.*, 1983). The protozoa *Tetrahymena pyriformis* grew best at 25 °C, but higher numbers of *L. pneumophila* were

present at 35 °C possibly due to enhanced multiplication because the protozoa were already stressed prior to infection. By coculture of cooling towers water with *Tetrahymena pyriformis* more strains of the *L. pneumophila* could be isolated than by traditional bacterial methods (Anand *et al.*, 1983, 1984).

Importance of cysts in protection against biocide treatment

The intracellular aggregation of *L. pneumophila* within cysts of *Acanthamoeba palastinensis* suggested that the bacteria could be protected from water treatment (Skinner *et al.*, 1983). Infection of two cooling tower derived trophozoites, *Acanthamoeba polyphaga* and *Acanthamoeba palestinesis*, with bacterial cells which reacted with *L. pneumophila* but could not be cultured, led to calculations of the number of bacteria present in amoebae (Rowbotham, 1986). It was calculated that 10, 000 *L. pneumophila* could be accommodated in large amoebae and large numbers of motile bacteria could be observed within the thick walled mature cysts.

Evidence for the role of cysts in protection from biocide of intracellular *L. pneumophila* was provided by Kilvington & Price (1990). Coculture of *Acanthamoeba polyphaga* with *L. pneumophila* produced infected trophozoites which could be encouraged to form cysts by addition of pH encystment media. A concentration of 75 mg/l free chlorine was then necessary to prevent amoebal protection for the bacteria, which was much greater than required to eradicate free bacteria.

Possible routes of infection

It was suggested that inhalation of infected amoebae may give rise to Legionnaires' disease (Rowbotham, 1980). An estimation of the number of bacterial cells in infected *A. polyphaga* was 326 large *L. pneumophila* and 1,320 small *L. pneumophila*, led to speculation that inhalation of infected amoebae was the main route of *L. pneumophila*

into the lung (Rowbotham, 1984). It was also proposed that Pontiac fever was due to hypersensitivity to amoebae and only mild infection by *L. pneumophila*. However, routine infection of amoebae by *L. pneumophila* (used as a method of assessment of virulence) provided an estimated number of intracellular bacteria which vastly differed from those suggested above (Vandenesch *et al.*, 1990). Microscopic investigation showed that 10-100 bacterial cells were present in vacuoles which occupied most of the amoebal cytoplasm and appeared as short rods.

Is intracellular growth the only mechanism?

If intracellular growth of *L. pneumophila* was obligate, then *L. pneumophila* would only grow in water systems when amoebae were present. In an attempt to provide evidence for the coexistence of *L. pneumophila* with amoebae, drinking water, whirlpool water, raw and treated waters were examined (Rowbotham, 1986b). Although *L. pneumophila* was found in 41% of water samples and amoebae were present in 68% of samples, they only coexisted in 27% of the samples. There was evidence that there was a spatial separation due to temperature, as amoebae were numerous at low temperature when 68% of samples contained amoebae but only 5% contained *L. pneumophila*. Whilst at higher temperatures *L. pneumophila* were more common and at 57°C samples contained *L. pneumophila* but amoebae were absent.

The relative importance of cyanobacteria, heterotrophic bacteria, free-living amoebae and ciliated protozoa on the growth of *L. pneumophila* was assessed using naturally occurring tap water cultures. Using successively smaller pore sizes Wadowsky *et al.* (1988) determined the effect of different sized growth factors. Unfiltered water provided maximum growth of *L. pneumophila* and then proportional reductions occurred with 5, 3, and 2 µm filtrates. Multiplication of *L. pneumophila* would not occur in the 1µm filtrate unless additional *Hartmanella* sp. were added indicating that

the amoebae were substituting the growth factor that had been excluded by filtration.

Samples of air and water from a shower were analysed for the presence of *L. pneumophila* and amoebae following 26 cases of Legionnaires' disease (Fields *et al.*, 1990). *Legionella pneumophila* was found in aerosolised droplets of <5µm from the shower and from several other sites. Amoebae were found in 86% of the samples which contained *L. pneumophila* and 22% of those samples which did not contain the bacterium. It was suggested that by controlling amoebal growth, *L. pneumophila* numbers could also be controlled.

1.6.2 Nutrient supply by other microorganisms

Photosynthetic organisms

Growth of *L. pneumophila* has not been solely attributed to intracellular growth in protozoa, as other microorganisms have been demonstrated to support growth extracellularly. Growth of *L. pneumophila* within an algal mat community was attributed to the utilisation of extracellular products of *Fischerella* sp. (Tison *et al.*, 1980). Several other green algae and cyanobacteria were demonstrated to support of *L. pneumophila*, including *Chroococcidopsis* sp., *Scenedesmus quadricauda*, *Synechocystis* sp., (Pope *et al.*, 1982) *Scendesmus* sp., *Chlorella* sp., and *Geocystis* sp. (Hume & Haan, 1984).

Growth of *L. pneumophila* with *Fischerella* sp. was attributed to slime containing protein and carbohydrate (Bohach & Snyder, 1983a). The initial decline in apparent numbers of *L. pneumophila* when cocultured with *Fischerella* spp. was due to adherence of the bacteria within the slime layer which bounded the cyanobacterial filaments (Bohach & Snyder, 1983b). Other cocultures showed similar modes of attached growth, in the case of *Chlorella* sp., *L. pneumophila* formed microcolonies in

the interstitial areas between algal cells and these were embedded within mucilage on the chlorophyta cell surfaces. (Hume & Haan, 1984).

In culture with cyanobacteria, *L. pneumophila* was shown to have a broader pH range than could be previously shown on media, the organism could grow at pH 6.9-8.5 and up to 45 °C (Tison *et al.*, 1980).

Growth with bacteria

Unsterile water was found to support the growth of *L. pneumophila* in the dark, indicating that non-photosynthetic organisms could provide nutrient to the pathogen (Yee & Wadowsky, 1982). *Flavobacterium breve* was then demonstrated to provide l-cysteine (an essential growth requirement) to *L. pneumophila* on solid media (Wadowsky & Yee, 1983). Satellite growth of *L. pneumophila* around several unidentified aquatic bacteria suggested that autotrophic bacteria from water systems could be capable of supplying nutrients to *L. pneumophila* (Wadowsky & Yee, 1985).

Environmental isolates of *Flavobacterium*, *Pseudomonas*, *Alcaligenes* and *Acinetobacter* spp. were all capable of supporting satellite growth of *L. pneumophila* on cysteine deficient media (Stout *et al.*, 1985). Clinical isolates of *Neisseria meningitidis* and *Haemophilus influenzae* sustaining *L. pneumophila* in a similar manner, but cysteine was not provided by *Escherichia coli*, *Klebsiella oxytoca*, *Candida albicans*, *Providencia stuartii*, *Pseudomonas aeruginosa*, *Morganella morganii*, *Staphylococcus aureus*, *Streptococcus pyrogenes*, and *Streptococcus pneumoniae* (Stout *et al.*, 1986).

Nutrient conditions had an essential effect on the ability of other microorganisms to support growth of *L. pneumophila*, since it was found that organisms which could encourage growth under some conditions failed under different environments (Toze *et al.*, 1990).

1.6.3 Inhibition by other microorganisms

Colony growth of *L. pneumophila* was shown to be inhibited by some respiratory flora (Carrington, 1979). *Streptococcus aureus*, *Klebsiella pneumoniae* a *Bacillus* sp. and a viridans *Staphylococcus* all prevented colony formation, although other species were not suppressive, including *Streptococcus bovis*, a *Corynebacterium* sp. and two further *Streptococcus* sp. Several bacterial isolates of human pharyngeal origin (*Streptococcus* sp., *Staphylococcus* sp. and *Micrococcus* sp) were found to inhibit growth of *L. pneumophila* on agar plates (Flesher *et al.*, 1980).

Aeromonas species, *Vibrio fluviialis*, *Pseudomonas vesicularis*, *Pseudomonas*-like strains and up to one third of the isolates from a distribution system were found to be capable of inhibiting *Legionella* sp. (Toze *et al.*, 1990). *L. pneumophila* was the least inhibited in comparison with other legionellae, which may explain the prevalence of *L. pneumophila* in drinking water systems.

In three cases of fatal Legionnaires' disease, *L. pneumophila* was absent from the area of the lung where *Aspergillus* hyphae already existed in the tissue, suggesting that antibiotic production by the *Aspergillus* may be one mode of inhibition (Chandler, 1981). This was supported by the suggestion that inhibition of *L. pneumophila* by *Pseudomonas aeruginosa* due to pyranin production during growth on agar media (Rowbotham, 1980,1986).

Endoparasitic *Bdellovibrio* spp., which are capable of using legionellae as their hosts, have been simultaneously isolated from water samples (Richardson, 1990). Although *Bdellovibrio* spp. are capable of attacking a broad range of Gram negative bacteria, the organisms may have a role in reducing legionellae populations in potable water.

1.7 POTENTIAL METHODS FOR CONTROL OF LEGIONELLA IN POTABLE WATER

1.7.1 Use of elevated temperatures

Pure culture work suggested that the use of elevated temperatures would be a suitable method for controlling *L. pneumophila* in water supplies (Stout *et al.*, 1986). Suspensions of *L. pneumophila*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were exposed to 60, 70 and 80°C to determine the decimal reduction times. All members of the *Legionellaceae* were rapidly killed when exposed to 50°C and higher temperatures did not greatly enhance the rate of killing.

A hospital hot water system was responsible for several cases of Legionnaires' disease when the operating temperature was reduced by 10 °C from 55-63°C (Palmer *et al.*, 1986). Reintroduction of the original operating temperature for the hot water was necessary to resolve the problem. Some attempts at controlling *L. pneumophila* by heating have been unsuccessful and additional methods have been required. *Legionella pneumophila* were found in 16% of hot water samples following a nosocomial outbreak of Legionnaires' disease, and after heat treatment of 60°C for 30 min *L. pneumophila* could be recovered (Snyder *et al.*, 1990). After 6 weeks of chlorination to 2 mg/l, 7% of samples still contained *L. pneumophila* indicating the problems of control are difficult to overcome when plumbing systems are already contaminated.

The problem encountered in many large, older buildings is that elevation of temperature of the whole system is difficult to achieve. Systems were often designed to operate at specific capacities, over time additional runs of pipe have been added, and this often exceeds the designed capacity. In addition, plans are not made which can result in a lack of information concerning the size and layout of the plumbing system and often results in sections of pipe containing static water which are not possible to heat treat.

Another reason for the low operating temperature of some hospital water systems is the deliberate use of low water temperatures to prevent scalding of patients. A self regulating heating element was designed for these type of situations, hot water supply was maintained at 45°C and then the device heated outlet water to 50°C. The whole plumbing system was colonised by *L. pneumophila* and although the device significantly reduced the numbers of *L. pneumophila* at the outlet *L. pneumophila* could not be eliminated from the shower water (Makin & Hart, 1991).

1.7.2 Use of chlorine in potable water

The susceptibility of pure cultures of *L. pneumophila*, *L. micdadei*, *K. pneumonia*, *E. aerogenes*, *E. coli* and *S. aureus* to chlorine was compared in sterile tap water (Kutcha *et al.*, 1983). All strains of *E. coli* and *K. aerogenes* were killed after only one minute contact with 0.2 mg/l free chlorine. *L. pneumophila* was less susceptible, requiring 8 and 60 min contact time at 21°C for 0.5 and 0.2 mg/l free chlorine respectively. Low temperatures and elevated pH resulted in losses in efficacy of chlorine and much greater contact times were then required. One method for overcoming the problem associated with high pH waters was the use of organic halamine instead of chlorine (Swango *et al.*, 1987). Use of organic halamines provided prolonged action and rapid kill times even at pH 9.5 with the additional benefit of reduced corrosion. Monochloramine has been shown to be less unstable than chlorine and able to penetrate biofilms (LeChevalier *et al.*, 1988). A model system using sterile water was able to show that both attached and planktonic *L. pneumophila* was more susceptible than *E. coli* to low levels of monochloramine (Cunliffe, 1990).

Chlorination of water entering a hospital to 1-2 mg/l free chlorine was successful in controlling *L. pneumophila* in cold water after 11 cases of Legionnaires' disease (Fischer-Hoch *et al.*, 1981). However, in hot waters, elevated temperatures were necessary to control the pathogen since chlorine was driven from solution at 45°C.

Unfortunately, control of *L. pneumophila* by chlorination was not always successful. After 24 cases of Legionnaires disease hospital cold water was chlorinated to 5 mg/l free chlorine and the hot water was maintained at 60°C after shock chlorination (15 mg/l free chlorine for 12 h) (Helms *et al.*, 1983). Two further cases of Legionnaires' disease indicated that the chlorine treatment had been unsuccessful in eradicating the pathogen. Similarly, treatment of cold water with 4 mg/l free chlorine only resulted in a reduction but not eradication of *L. pneumophila*, with 12.5% of samples being positive (Baird *et al.*, 1984). A possible reason for biocide failure, could be an underestimation of the concentration required to kill *L. pneumophila*. Agar passaged strains were demonstrated to lose their resistance to biocide when compared with tap water passaged strains (Kutch *et al.*, 1985), indicating that test methods used for biocide evaluation should be carefully controlled.

The use of higher chlorine levels was found to be more effective in controlling *L. pneumophila*. The number of samples containing *L. pneumophila* was reduced from 21% to <1% after the cold and hot water systems were treated with 7-8 mg/l free chlorine (Massanari, 1984). During a survey, *L. pneumophila* was not detected in water samples when free available chlorine residuals were above 7.5 mg/l (Tobin *et al.*, 1984).

1.7.3 Use of ozone in potable water

The suitability of ozone as a suitable disinfection method for water supply was tested in a disused wing of a hospital where the water system was found to be contaminated with *L. pneumophila* (Edelstein *et al.*, 1982). Pure culture work indicated that 0.47 mg/l were sufficient to kill suspensions of *L. pneumophila*. Although *L. pneumophila* numbers were reduced they were still recoverable from the system when an ozone level

of 1.0 ± 0.3 mg/l were used. Results were also inconclusive since the untreated, control system also had a reduction in the numbers of *L. pneumophila*.

1.7.4 Use of UV light in potable water

The use of chlorine, heat, UV light and ozone were compared using a model system (Muraca *et al.*, 1987). Broth grown *L. pneumophila* was suspended in unsterile water and recirculated around a model system. At 25°C 4-6 mg/l free chlorine reduced *L. pneumophila* by 5-6 logs after 6h, at 43°C 120% more chlorine was required. Use of 1-2 mg/l ozone or UV light disinfection ($30\,000\ \mu\text{W}\cdot\text{s}/\text{cm}^2$) produced a similar reduction in the numbers of the pathogen with the additional advantages of being unaffected by temperature or turbidity. Heat was the most successful method for the eradication of *L. pneumophila* from the system.

UV light was successfully used to control *L. micdadei* in a hospital where there had been an outbreak of Legionnaires' disease (Farr *et al.*, 1988). After hyperchlorination of the water, a UV lamp was fitted ($30\,000\ \mu\text{W}\cdot\text{s}/\text{cm}^2$) resulting in a reduction of *L. micdadei* to undetectable levels in water samples. When the lamp was removed, *L. micdadei* were recovered indicating that the UV lamp had been successful in controlling the pathogen.

1.8 POTENTIAL METHODS FOR CONTROL OF *L. PNEUMOPHILA* IN COOLING TOWERS

1.8.1 Use of biocides in cooling towers

Prior to the discovery of *L. pneumophila* as a pathogenic member of cooling tower communities, the sole function of biocides was to limit biofouling. Biocide treatment

and cooling tower maintenance was viewed from an engineering perspective. Biocides were used to ensure heat transfer efficiency and prevent damage to tower structure by the accumulation of fouling material. When *L. pneumophila* was recognised as an important pathogen which could be disseminated via cooling tower drift, little was known of the suitability of biocides then available for use in controlling the pathogen. Examination of cooling towers and air-conditioning systems showed that those treated with chlorine or non-volatile organic biocides contained at least 10^6 /l *L. pneumophila*, with towers treated with 1.0-1.5 mg/l free chlorine having the lowest numbers. Air-conditioning systems that had been shock chlorinated and then maintained with residual chlorine at 14 mg/l were found to contain 10^5 /l *L. pneumophila* (Fliermans *et al.*, 1982).

Suitability of a range of available biocides at the suggested concentrations were compared in cooling towers several of which containing *L. pneumophila* and amoebae (Kurtz *et al.*, 1982). Only one biocide, a biodegradable chlorinated phenolic thio-ether with an antifoaming agent, was partially effective against *L. pneumophila* and activity could not be maintained. Two other biocides, dimethyl-didecyl ammonium chloride mixed with tributyltin oxide, and sodium di-chlor-isocyanurate were ineffective against the pathogen.

The failure of biocides to control *L. pneumophila* could, in part be attributed to the methods employed in selecting biocides and determining doses. When biocides were initially screened, a suitable culture medium for *L. pneumophila* was still in development; *L. pneumophila* could only be isolated following culture in eggs or lung tissue so environmentally derived strains were not used. The role of the microbial community in the proliferation of *L. pneumophila* was not recognised and so studies used *L. pneumophila* suspended in sterile water for testing biocides. Other factors which were not included in laboratory studies were the level of organic material in cooling towers, the operating temperatures of cooling towers and the effect of other

chemical additions on biocide activity (Skaliy *et al.*, 1980). However, laboratory methods allowed rapid screening of the most suitable biocides with the additional benefit of a guide to the minimum dosing requirements. For example, both calcium hypochlorite and a combined biocide (didecyldimethyl-ammonium chloride with isopropanol) were both effective against *L. pneumophila* in sterile water after 3h contact time at concentrations of 3.3 mg/l and 72 mg/l, respectively (Skaliy *et al.*, 1980).

Some authors advocated cooling tower trials as the only option for the determination of biocide suitability, due to the failure of laboratory based trials to accurately determine the required biocide concentrations. One cooling tower was unsuccessfully treated with 1-bromo-3-chlor-5, 5-dimethylhydantoin at the manufactures' suggested dose of 0.2-0.5 mg/l and at a higher dose of 1.5-2.1 mg/l. Whereas, chlorine was demonstrated to be a suitable inhibitor of *L. pneumophila* in cooling towers at a concentration of 2.0 mg/l free chlorine (Fliermans *et al.*, 1984). Laboratory testing systems have however, also provided some valuable information despite possible criticism. The testing of biocide against *L. pneumophila* in physiological saline demonstrated that *N*-alkyl 1,3-propanediolamine and methyl bis-thiocyanate required much larger concentrations than those suggested by the manufacturer, whereas alternatives (*N*-alkyl dimethyl benzyl ammonium chloride and bis tri-*n*-butyltin oxide) were effective at concentration a 25 times less than those suggested.

Algae are capable of high biomass production and are a source of nutrient for growth of *L. pneumophila* in cooling towers, consequently control of these organisms is essential for good maintenance of cooling towers. Axenic cultures of *Chlamydomonas*, *Chroococcidiopsis*, *Fischerella*, *Scenedesmus* and *Synechocystis* spp. were tested for susceptibility against biocides. Several were efficient at 1/10 the suggested doses but calcium hypochlorite with 1,3,4,6-tetrachloroglycoluril, phosphoric acid with nonyl phenoxypoly(ethylenoxy)-thanol iodine complex and 2,2-dibromo-3-nitrilpropionamide were not effective. In actual cooling towers however, the eradication

of *L. pneumophila* and algae was difficult to achieve, suggesting biocide efficacy had been overestimated (Soracco *et al.*, 1983).

1.8.2 Use of ozone in cooling towers

Survival of *L. pneumophila* was compared in a cooling tower treated with 0.6-1.8 mg/l ozone and with a cooling tower treated with a weekly dose of 138ppm disodiumcyandithioimido carbonate and potassium N-methyl dithiocarbamate mixture. Ozone was found to be more effective than the biocides, with reductions in mean plate count of total bacteria and number of *L. pneumophila* as enumerated by combined INT/DFA staining (Pope *et al.*, 1984).

Survival of *L. pneumophila* suspended in sterile water was compared against ozone, hydrogen peroxide and chlorine (Dominique *et al.*, 1988). At 45°C and with 30 min contact time, a 99% reduction of *L. pneumophila* required 1000 µg/l hydrogen peroxide. In contrast, at both 25 and 45°C, 0.1-0.3 mg/l ozone was sufficient to inactivate *L. pneumophila* after only 5 min contact time. Chlorination at 0.4 mg/l with 5 min contact time was also effective. The major advantage of ozonation in water treatments is that the by-products are non toxic (water and oxygen).

1.8.3 Use of UV light in cooling towers

The use of bronopol (2-bromo-2-nitropropane-1,3-diol), isothiazolone (methylchloro/methylisothiazone) and UV light sterilisation were compared for efficacy in controlling *L. pneumophila* in cooling towers (Yamamoto *et al.*, 1991). The two biocides were found to successfully eradicate the *L. pneumophila* but the cooling tower treated with UV light was found to contain 10-100 *L. pneumophila* ml⁻¹. Survival of the pathogen was attributed to accumulation of dirt on the lamp, despite the presence of a teflon

coating which should have prevented this accumulation. The use of UV light as the sole method for controlling *L. pneumophila* is not a "fail-safe" mechanism for protecting a system. Bacteria must pass through the UV loop to be disinfected, those bacteria which are growing on the surfaces of the tower, are unaffected by the UV light and are possibly growing in a situation of reduced competition for nutrient. If the lamp becomes too dirty to operate efficiently or fails completely, the whole system is unprotected.

1.9 PROBLEMS OF CONTROL OF LEGIONELLAE IN WATER SYSTEMS

1.9.1 Resistance of protozoa to biocides

The resistance of amoebal cysts to biocide has been recognised as one survival mechanism for their persistence in water systems. Cysts of *Naegleria fowler*, *N. gruberi*, *A. culbertsoni* and *A. polyphaga* were able to withstand 4 mg/l free chlorine for 4h, with pathogenic species being more resistant than non-pathogenic species (Jonckheere, 1976). *Naegleria gruberi* cysts have also been demonstrated to be less susceptible to chlorination than bacteria and viruses (Spoul *et al.*, 1983).

Enhanced survival of *L. pneumophila* with chlorination was demonstrated by intracellular protection within *Tetrahymena pyriformis*. A 50% increase in the resistance of *L. pneumophila* to 1 mg/l free chlorine followed ingestion, and at 4 mg/l free chlorine a 99% reduction in bacterial numbers required a contact time of 75 min (King *et al.*, 1988).

The survival of intracellular *L. pneumophila* within *A. polyphaga* during exposure to chlorine has been demonstrated. Infected trophozoites were encysted and were able to tolerate chlorine concentrations of up to 50 mg/l. The *L. pneumophila* were protected

from the chlorine concentration and remained viable despite excessive chlorine levels (Kilvington and Price, 1990). The amoebae was also found to protect *L. pneumophila* from treatment using benzisothiazolone or 5-chloro-*N*-methylisothiazolone (Barker *et al.*, 1992). Polyhexamethylene biguandi was effective against intracellularly grown *L. pneumophila* and amoebae at a concentration of 10 µg/ml.

1.9.2 Resistance of biofilms to biocide

The comparative resistance of biofilm bacteria compared with planktonic microorganisms has been recognised for many years (Costerton *et al.*, 1987). In the industrial field for example, the plugging of oil wells is attributed to the survival and growth of bacterial biofilm populations in the presence of biocide, despite the fact that the biocide concentrations would appear sufficient to control the same species of microorganisms in planktonic culture (Rueska *et al.* (1982) and Cusack *et al.* (1988)). In the medical field, *Pseudomonas aeruginosa* present on medical implants has been shown to be more resistant to antibiotics (Nickels *et al.*, 1985). Interestingly, if biofilm cells are resuspended the acquired resistance to biocide treatment is reduced (Anwar *et al.*, 1989).

The increased resistance of biofilm bacteria has been partially attributed to the inability of the biocide to penetrate the microorganisms which grow adherent to the surface in microcolonies enclosed within a glycocalyx (Costerton *et al.*, 1984). It has also been postulated that the bacterial species present on the surface and the nature of the substratum influence the degree of antibiotic resistance (Gristina *et al.*, 1989). A major influence is now considered to be the growth rate of the populations of microorganisms in the biofilms, which has been demonstrated to be lower than in the planktonic phase (Brown *et al.*, 1988). Since some antibacterial agents (eg β-lactams) are dependent on bacterial growth for their action, these slow growing biofilm bacteria are not susceptible to their actions (Tuomanen *et al.*, 1986).

Populations of microorganisms in drinking water distribution systems were able to survive despite chlorination (Ridgeway and Olsen, 1982). This was postulated to be due to selection of chlorine resistant microorganisms and the presence of organisms in aggregates or attached to particles. Attached Actinomycetes were shown to be chlorine tolerant and able to survive exposure to 10 mg l⁻¹ free chlorine for 2 min. Less tolerant organisms which were killed at 1.0 mg l⁻¹ free chlorine included those identified as *Corynebacterium/Alcaligenes*, *Klebsiella*, *Pseudomonas/Alcaligenes*, *Flavobacterium/ Moraxella* and *Acinetobacter* spp. Attachment also provided protection from chlorination for *Enterobacter cloacae* (Herson *et al.*, 1987). Greater ability to survive the chemical treatment was confirmed when a greater length of time was available for attachment.

Adherent *L. pneumophila* were also found to be more resistant to 2-bromo-4-nitropropane 1,3-diol and an isothiazolin-containing biocide than the planktonic populations (Wright *et al.*, 1991). This was demonstrated in a pure culture biofilm model where recirculated artificially hard water was supplemented with 10 % algal extract. *L. pneumophila* was inoculated at a concentration of 10⁸ in a total volume of 900 ml. The biofilms were allowed to develop on PVC and douglas fir coupons held in a Robbins Device for 14 days and biocide was added.

1.10 MATERIALS IN CONTACT WITH POTABLE WATER

1.10.1 Elastomeric components of water systems

For many years, it had been recognised that several species of *Actinomyces* and *Pseudomonas* existed on the surfaces of latex components of water systems and that these caused biodeterioration. Oxidative decomposition of 0.2-0.5 mg from 250-300mg

of rubber in 10 days at 25°C, suggested that 3-10 % of the material would be degraded over one year. Decomposition by mixed microbial populations was more rapid than by pure cultures as measured by oxygen consumption, weight loss and growth of the microbial consortium. Some synthetic rubbers were also susceptible to microbial attack (Zobell & Beckwith, 1944).

Rubber samples from over fifty corroded sites in the Netherlands were shown to be degraded by *Streptomyces* (Leeflang, 1953) and test strips of rubber materials were shown to be degraded by pure cultures of the *Streptomyces* in batch systems. Test strips of natural rubbers were degraded at 25°C by flowing unchlorinated tap water over the surfaces and the rate of decomposition was dependent on the presence of antioxidants and the curing methods used. The synthetic compounds nitrile, neoprene, ethylene-propylene and styrene-butadiene rubbers were more resistant to biodegradation (Leeflang, 1968). Natural rubber gaskets used for sealing asbestos-cement pipes were also degraded by two species of *Streptomyces* and caused widespread failure of rubber components of pipelines in the Netherlands (Rook, 1955).

The examination of water and sewage lines led to the isolation of actinomyces (*Streptomyces litmanii* and *Streptomyces fulviridis*) and thiobacilli at sites of degradation of natural rubber and these were thought to be important in biodeterioration of rubber components in water systems (Hutchinson *et al.*, 1972).

Rubber materials which contain accelerators (for example tetramethyl thiuram disulphide (TMTD) or monosulphide (TMTS)) were found to inhibit microbial degradation (Colbourne and Ashworth, 1986). Materials which included them in the formulation were able to support biofilms suggesting that each rubber formulation should be tested and not each individual component, since the presence of a particular additive did not necessarily constitute suitable composition.

The extracellular enzyme responsible for the rubber degrading activity of a *Xanthomonas sp.* was isolated from a strain grown on natural rubber by Tsuchii and Takeda, (1990) who showed that the products were isoprene oligomers.

Leaching of low levels of zinc, manganese, copper, iron and magnesium from rubber stoppers used for feeding animals was demonstrated, although the levels were considered toxic for feeding animals, metals essential for enzyme production in bacteria may have been supplemented by these materials (Kennedy & Todd, 1991). Bacterial growth or leaching of carbon substrates and the impact of these on the quality of water was not considered.

1.10.2 Copper pipe

Domestic water systems have been traditionally constructed using copper, this was known to metabolically injure the bacteria causing inhibition and decreased recovery on solid media (MacLeod *et al.*, 1966). However, inhibition by copper was not universal to all bacteria. Several species of bacteria from drinking water systems have been shown to be tolerant of high copper concentrations including *Micrococcus sp.*, *Acinetobacter sp.*, *Pseudomonas sp.* and *Alcaligenes sp.* and this correlated with multiple resistance to lead, zinc and antibiotics (Calomiris *et al.*, 1984). In addition, loss of culturability of bacteria isolated from drinking water supplies was shown to be prevented, for some species, by the addition of Na₂ EDTA to chelate the copper (Versteegh, 1989).

Attachment appeared to play a role in tolerance to copper since bacteria attached to sediment were found to be more resistant to copper than bacteria associated with algae (Said and Lewis, 1991). The MIC (minimum inhibitory concentration) of copper in sediments was 1.2 µM and in aufwuchs (algal floating mats) was 0.42 µM. Bacterial biofilms are known to develop on the surface of copper surfaces up to 55°C and may

result in microbially induced corrosion (Walker *et al.*, 1990).

1.10.3 Plastic pipe

During the 1950's there was a developing interest in the use of plastic pipes for potable water supplies because of the resistance to corrosion, light weight and ease of installation. Polyethylene, polyvinyl chloride, rubber modified polystyrene and cellulose acetate butyrate were evaluated for suitability for use in potable water systems (Tiedeman 1953, Tiedeman & Milone 1958). The materials had no significant effect on taste or odour, were sterilisable using chlorine and were not toxic to rats. Problems of leaching of water-insoluble solvents used to seal joints of the cellulose acetate butyrate could be eliminated by blowing air through the system, flushing with detergent and then clear-water flushing. In addition, water used for testing suitability of the material was found to contain fungus after contact with the material and soil weathered samples were found to support biofilm. Retrospectively, these observations may have indicated that microbial growth could be encouraged by these new materials and detrimentally effect water quality.

1.10.4 Components used in plastic manufacture

The major components of plastics is a resin or a cellulose derivative. Plasticisers (usually high-boiling point, low volatility esters) are added to increase the flexibility. Most commonly these are diethyl and dimethyl glycol pthalates, but if the plastic is required to withstand low temperatures then di-2-ethyloxy pthalate is used. PVCc requires the highest proportion of plasticiser.

All polymers except rigid PVC and fluorocarbons require flame retardants, which may be triphenyl phosphate (which is also a plasticiser), bromine, chloride, antimony and phosphorus compounds.

PVC requires lead salts and stearates to prevent degradation and emission of hydrochloric acid during the high temperatures required for processing manipulations. Fillers are cheap substances used to increase bulk, provide strength or metal properties. These include alkyl compounds of tin, but if the finished product is to be transparent zinc or barium-cadmium is used. Mould lubricants are also required during processing to prevent extruded pipes from sticking and these are soaps of heavy metals.

Low density polyethylene must be protected by antioxidants (commonly phenols containing esters or phosphorites) since the large numbers of side chain junctions are vulnerable to oxidation. UV light initiated oxidation is prevented by the addition of carbon black, phenols and naphthols containing sulphur. Polypropylene is extremely susceptible to oxidation, especially at higher temperatures and so phenol and sulphur containing compounds (such as distearyl thiodipropionate) are used as antioxidants.

All polymers except rigid PVC and fluorocarbons require flame retardants. These are bromine, chlorine, antimony and phosphorus compounds.

1.10.5 Leaching of substances from plastic pipes

Prior to use in water systems, plastics were extensively tested for possible toxic effects. The leaching of toxic stabilisers from unplasticised polyvinyl chloride (PVCu) was assessed by evaluating lead levels in distribution systems and by extraction of lead from pipe sections. No actual systems exceed the maximum permitted by the Water Supply (Water Quality) Regulations (1989), of $50 \mu\text{g l}^{-1}$. However, a test rig had concentrations of lead exceeding the limits when new pipe was fitted but this decreased to an acceptable level after a few days. It was suggested a flushing stage at the end of the manufacturing process may eliminate the problem (Packham, 1971).

The interior surface of PVCu pipe was found to contain a lead-rich layer (of approximately 30mg Pb m² of pipe). The lead could be removed from this layer by acid or alkaline reagents. Slow diffusion of lead could be removed from the bulk phase at a rate of 0.2 mg Pb m⁻² each week (Packham, 1972). Low levels of tin, cadmium and magnesium could also be released into water from PVC pipe (Dietz *et al.*, 1979).

Another potential leachate of PVC pipe is the residual monomer of vinyl chloride. This was estimated to diffuse out of pipes containing 22 mg kg⁻¹, at a rate of 5µg kg⁻¹ of vinyl chloride monomer in water extracts (Berens and Daniels, 1981). Monochlorethylene (vinyl chloride) has been shown to be degraded by *Nitrosomonas europaea* (Vannelli *et al.*, 1990), by *Actinomycetales* (Phelps *et al.*, 1991) and by *Methylosinus trichosporium* (Tsien *et al.*, 1989). Vinyl chloride has also been shown to be capable of being mineralised in groundwater by Davis and Carpenter (1990). Chloroform and carbon tetrachloride (tetrachloromethane) were extracted from rigid PVC and PVCc pipe into water at very low levels of the µg l⁻¹ range by Desrosiers and Dunnigan (1983). These authors thought that more contamination was likely to occur from the solvent cement used in the jointing of the plastic pipes. Carbon tetrachloride was also degraded by *Nitrosomonas europaea* (Vannelli *et al.*, 1990).

The ability of bacteria to degrade plasticised PVC was shown to occur when test strips were inoculated by isolated bacterial species in mineral salts medium (Booth *et al.*, 1968). A combination of two *Pseudomonas* spp. and a *Brevibacterium* sp. produced the most rapid weight loss in the test strips. This loss was attributed to utilization of plasticiser (di-iso-octyl sebacate, DIOS). Up to 65% of the plasticiser was utilised in two weeks. The authors found that the *Pseudomonas* spp. were further degrading the by products from the degradation of the plasticiser by the *Brevibacterium* sp.

Another plasticiser, di-*n*-butylphthalate can be degraded aerobically (Englington and Barnes, 1978) and anaerobically (Bernckiser and Ottow, 1982). Under anaerobic conditions the plasticiser was shown to be metabolised by a denitrifying strain of *Pseudomonas pseudoalcaligenes*. The butanol moiety served as the essential carbon source for both growth and denitrification, since nitrate was the sole electron acceptor.

Following slight nutrient enrichment of mineral salts medium with yeast extract, a species of *Corynebacterium* and a strain of *Pseudomonas aeruginosa* were able to degrade polyester polyurethane in 14 days (Kay *et al.*, 1991).

1.10.6 Testing suitability of materials for use in water supplies

A wide range of materials were evaluated for their ability to encourage growth of microorganisms using a naturally occurring inoculum in tap water. The results showed that 68% of rubber materials, 60% of glass reinforced plastics, 25% of other plastics and 50% of jointing materials encouraged microbial growth. It was concluded that many of the materials used in water supplies were likely to promote microbial growth (Burman & Colbourne, 1977). This work led to the development and introduction of a testing method for materials in contact with potable water, BS 6920.

Materials used in contact with potable water are subject to British Standard BS6920. Conformity to this standard ensures that the materials do not impart unacceptable taste or odours, toxic chemicals or encourage the growth of microorganism. For microbiological growth the method employs a simple, batch test where the material is placed in a specially cleaned, airtight, glass container. Chlorine free, chemically defined water and an inoculum of naturally occurring flora are added. Incubation is at 30°C in the dark and the water is replaced by fresh water twice a week for a total of 8 weeks. Counts of specific, planktonic bacteria and measurement of dissolved oxygen are used

to determine suitability of the material. Results are compared with a positive control of paraffin wax and a negative control of glass. Paraffin wax acts as an insoluble nutritive surface for biofilm formation. The regression line of the dissolved oxygen difference is used to rank the material in order of its ability to allow growth of microorganisms by supplying nutrients. Acceptable plastics and elastomeric surfaces give mean Dissolved Oxygen Differences of 1.0 to 2.0 mg/l, paraffin wax is 5.6 to 6.0 mg/l, approved rubbers have 0.1 to 1.3 mg/l and copper is 0.25 to 0.37 mg/l. A value of greater than 2.3 mg/l is unacceptable and the material not approved for use.

1.11 AIMS OF THIS STUDY

There has been little work comparing the colonisation of materials following the introduction of the BS6920 testing procedure. Pedersen (1990) evaluated biofilm development in chlorinated, unsterile, tap water on the surface of stainless steel and on PVC pipe, and found no difference in the colonisation of the materials following exposure for over 5 months. Comparison of biofilm development was by staining the microorganisms with acridine orange and counting fields of view, so the data shows that total biofouling may be similar, the nature of the populations on the materials was not investigated.

Laboratory culture methods, utilising complex media, are unsuitable for investigating the complexity of an aquatic consortia growing as biofilms on various plumbing materials. The technique of continuous culture has proven invaluable for ecological studies due to its close control of environmental parameters, specificity and reproducibility (Keevil *et al.* 1989a, West *et al.*, 1989).

The aims of this study were to develop a model of a water system for growth of *L. pneumophila* in an appropriate mixed community. The use of the model for the ecological study of *L. pneumophila* was to be evaluated and the relevance of the results to water environment, in particular, the implications for the design and operation of water systems for the prevention of infection with *L. pneumophila* was to be considered.

The initial aims were therefore :-

- 1) to establish growth of a suitable microbial consortium in tap water;
- 2) to ensure that biofilm and planktonic growth was reproducible;

The system was then to be used to investigate:-

- 3) the colonisation of glass surfaces by the mixed microbial population. In particular, the colonisation by *L. pneumophila* to provide a information on the growth

of the pathogen within the community;

4) to compare the effect of plumbing materials on biofilm development and inclusion of *L. pneumophila*;

5) to determine the influence of temperature on the growth of *L. pneumophila* and the microbial consortium.

6) finally, to determine suitable control measures for biofouling and growth of *L. pneumophila* in cooling towers.

CHAPTER 2

MATERIALS AND METHODS

2.1 THE MODEL SYSTEM

A model plumbing system was developed which used tap water as the growth medium with no added supplements for inoculated microorganisms. Although pure culture or biculture work has been useful in the mathematical modelling of populations, it is unsuitable for modelling complex communities where interactions occurring between different species of micro-organisms influence the composition of the resulting biofilm. In addition, mixed culture modelling is essential as legionellae are incapable of growth in sterile, unsupplemented water (West *et al.*, 1989) and plumbing systems are colonised by numerous species of bacteria. The model system was designed so that the fluid velocity in the vessel was between $1\text{--}2\text{ m s}^{-1}$, so maintaining the Reynolds number well below the transition zone between laminar and turbulent flow, required to keep the chemostat model within the rates of flow that exist in plumbing pipes (Perry & Green, 1984).

2.1.1 Nutrient supply

The model required a constant supply of sterile tap water to provide nutrient for the growth of the microorganisms within the consortium. The tap water used in the study was supplied from a domestic cold water source which had been softened. In previous trials this water was shown to support the growth of *L. pneumophila* (West *et al.*, 1988). The water was supplied to the laboratory on a weekly basis, filter-sterilised using a 142mm, $0.22\mu\text{m}$ pore size nylon filter as soon as possible, so as to prevent the depletion of nutrients by the growth of contaminating micro-organisms, and stored at 4°C . Ten litres of water was pumped through an autoclaved filter housing (Sartorius) containing two Nucleopore membranes and aseptically transferred into a 10l Nalgene

media bottle. Water sterilised this way has been shown to remain chemically unchanged (Colbourne *et al.*, 1988).

2.1.2. Inoculum

The inoculum for the chemostat was sludge from the bottom of a calorifier implicated in an outbreak of Legionnaire's disease and contained an indigenous population of *L. pneumophila* serogroup 1 Pontiac with a diverse population of bacteria, amoebae and protozoa. To avoid artificial selection of microorganisms for inoculation, microorganisms had not been subcultured prior to inclusion into the chemostat.

2.1.3. Chemostat model

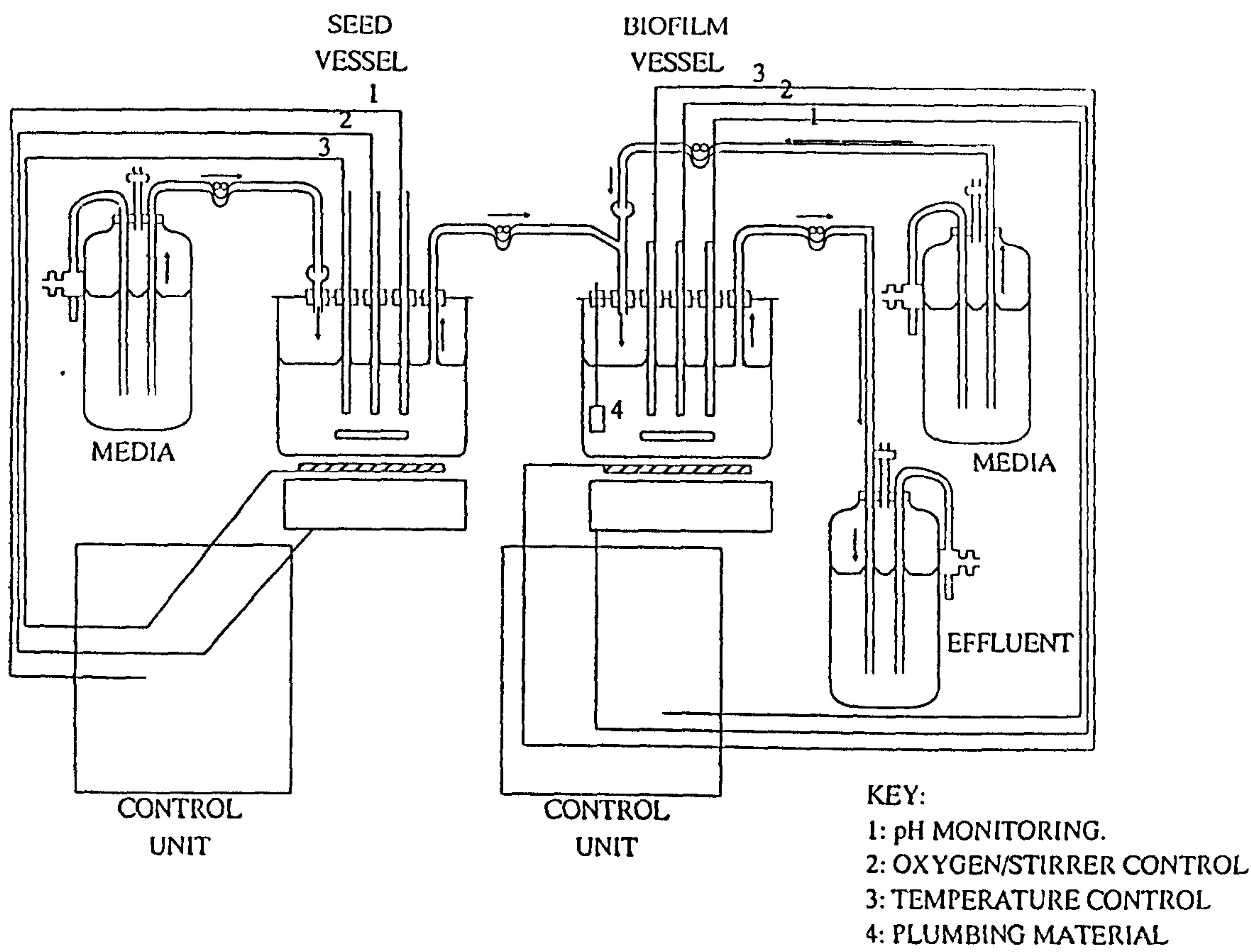
The model system was designed to reproduce the conditions that occur in a real water system. It consisted of three glass vessels linked in series, growth medium was pumped to the first two vessels and waste from the third vessel was pumped into an effluent bottle (Figure 2.1). Temperature, pH and oxygen probes were inserted through the top plate of each vessel into the culture and were connected to process control units. Heat was provided by an external, electrical heater pad located below the vessels. Agitation was achieved by the use of an external, magnetic-drive stirrer with the magnetic stirrer situated in the culture (Figure 2.2).

The first vessel represented a storage tank and the second vessel modelled the distribution system. To conserve the microbial population in the first vessel environmental conditions were kept constant. Culture from this vessel was constantly supplied to the second vessel where test materials were immersed. The model was housed in a Class 3 safety cabinet so that staff would be protected in the case of equipment failure

The first vessel had a retention volume of 500 ml, the flow rate of sterile water into the vessel resulted in a dilution rate of 0.05 h^{-1} . When the retention volume was exceeded, the effluent was pumped via a weir system into the second vessel. This second vessel had additional sterile water pumped into it, along with the effluent from the first vessel, maintaining a total dilution rate of 0.2 h^{-1} . The effluent from the second vessel was pumped via a weir into the third vessel. By selection of only silica glass, titanium and silicone tubing in the construction of the system, modification of the microbial consortium due leaching of metal ions or nutrient from materials was prevented.

The environmental parameters of the chemostats were controlled using Anglicon microprocessor control units (Process Systems, Newhaven) linked to a PC computer. The temperature of the vessels was maintained at $30.0 \pm 0.1^\circ\text{C}$ using Proportional Integral Derivative (P.I.D.) controllers. The temperature was measured using a glass temperature probe inserted into the aqueous phase of the vessel and the temperature was corrected using an external heater pad. The glass galvanic oxygen electrode was temperature compensated and the dissolved oxygen tension (DOT) was maintained at $20 \pm 0.5 \%$ via proportional control of the stirrer speed. The pH and Eh of the vessels were monitored through out the experiments.

FIGURE 2.1 : Diagram of the two stage chemostat biofilm model.

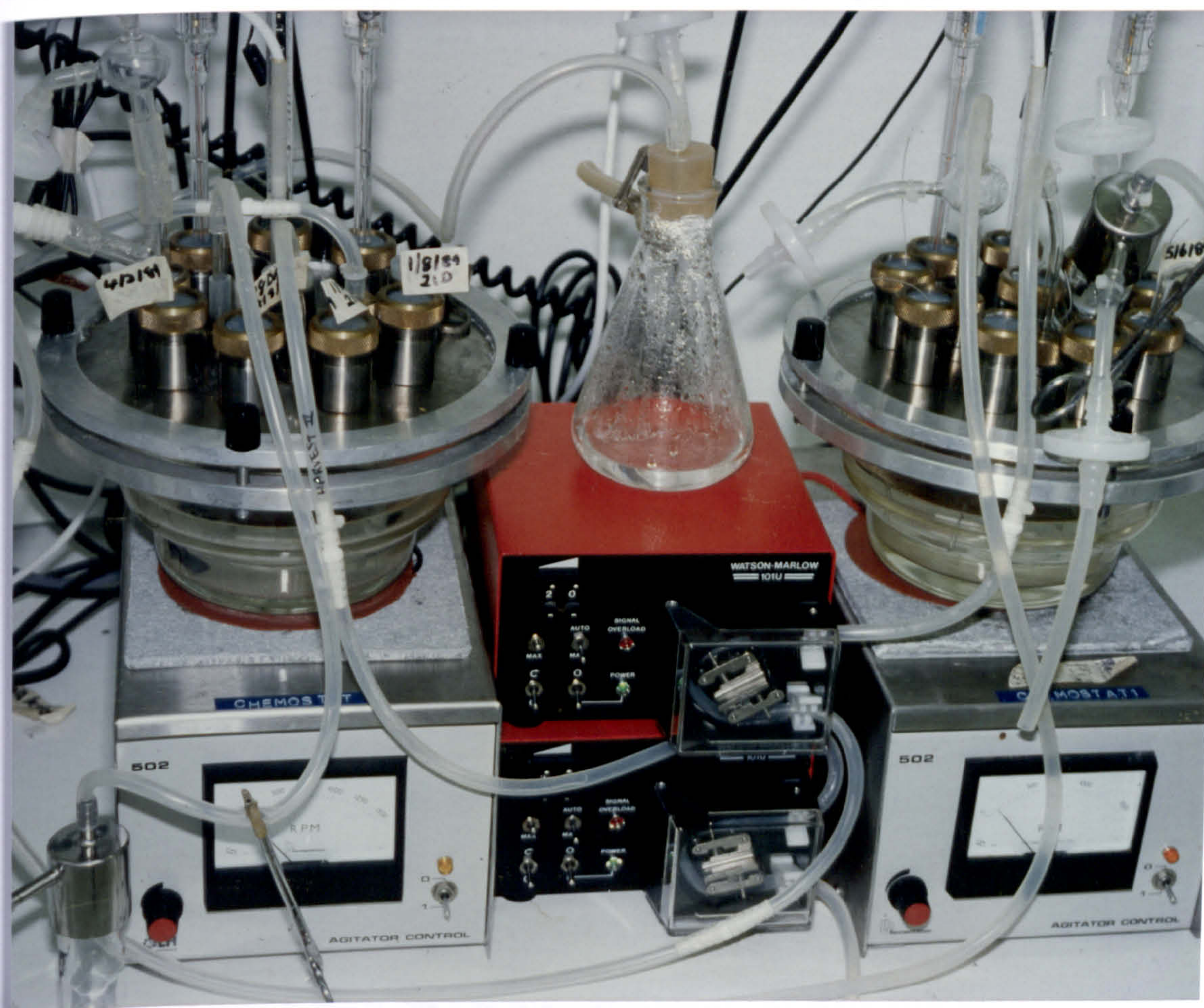


2.2. THE BIOFILM MODEL

2.2.1 Generation of biofilms

The materials used in the trials are as follows:

FIGURE 2.2 : Photograph of the actual chemostat model.



2.2. THE BIOFILM MODEL

2.2.1 Generation of biofilms

The materials used in the trials included copper, latex, ethylene-propylene, polybutylene, polypropylene, polyethylene, uPVC and cPVC. The plastic and copper plumbing tube was cut open to make test coupons of 1 cm² and a 1mm hole was drilled near one end so the coupons could be suspended into the chemostat on titanium wires. The materials were suspended from the wires in clusters, alongside the control glass surfaces, and then cleaned with acetone to remove any oil and dirt from the cutting process. The tile assemblies were put into glass bottles containing sterile water and heat sterilised by autoclaving. There was no visible effect of the heat on the plastic surfaces.

For each experiment the material and the control glass coupons were immersed into the aqueous phase of the second chemostat at day 0. The coupons were removed after 1, 4, 7, 14, 21 and 28 days from the onset of the experiment so that the development of biofilm could be assessed. Biofilm was aseptically removed from the inside surface of the pipe material using a sterile dental probe. The biofilm was resuspended in 1.0 ml of sterile water and vortexed in order to disperse the microorganisms. Development of the biofilm was assessed microbiologically as described in section 2.3.

2.2.2 Biofilm production and stability

Coupons were inserted into the chemostat vertically so that bacterial cells or clumps of cells did not fall onto the surface by gravity (as is the case when corrosion in pipes is caused in horizontal pipes by the accumulation of sediment). Care was taken to ensure that the whole surface of the material was immersed in the water and that the inside section of the pipe was facing towards the centre of the vessel. This ensured that the

surfaces did not come into contact with the stirrer which would dislodge biofilm and that the biofilms were exposed to equal shear forces being equal distances from the stirrer.

The sections of pipe were very cautiously removed from the chemostat after biofilm had formed, especially in removing the material through the vessel port. After removal of the material supporting the biofilm from the chemostat all samples were treated in exactly the same manner. The material was washed by complete immersion into sterile water with gentle movement for 15 sec to ensure that all unwanted planktonic bacteria were removed. The biofilm was then removed by scraping the whole surface methodically with a sterile dental probe and then immersed in sterile diluent. The resulting bacterial suspension was mixed with a vortex mixer at high speed for 60 sec in order to disperse the biofilm. Microscopy showed that some small clumps were still evident but these could not be dispersed further with vortexing.

During the trial, samples of the planktonic (aqueous) phase were aseptically removed via a sample port and bacterial numbers assessed in the same manner as the biofilms.

2.3. MICROBIOLOGICAL ASSESSMENT OF BIOFILM AND PLANKTONIC SAMPLES

2.3.1 Processing of biofilm and planktonic samples

The composition and concentration of microorganisms within the planktonic phase were assessed throughout each material trial. The water or resuspended biofilm sample was vortexed to disperse the microorganisms, serially diluted into sterile water and then inoculated onto various selective and non-selective agar media. Total microbial populations were enumerated using low nutrient R2A medium (Reasoner & Geldrich, 1985) to avoid substrate shock. Buffered charcoal yeast extract (BCYE) agar (Pasculle

et al, 1984) was used to culture the more fastidious bacteria including legionellae. BCYE medium was supplemented with glycine, vancomycin, polymixin and cycloheximide to produce GVPC agar, a selective medium for legionellae (Dennis *et al*, 1984). All media was prepared as described by the authors.

All plates were incubated at 30°C for seven days prior to counting the colonies that grew. One plate of each medium was selected for evaluation of population profiles.

2.3.2 Heat treatment of biofilm and planktonic samples

Planktonic and biofilm samples were inserted into a water bath at 50°C for 10min. Following removal the sample was mixed using a vortex mixer and immediately 0.1ml of sample was spread onto the BCYE and GVPC plates. Both heated and non-heated portions of the samples were plated.

2.3.3 Counting procedures

After incubation for 7 days at 30 °C agar plates were examined for the growth of microorganisms. The total flora was determined by the growth on R2A medium by counting the number of colony forming units (cfu) on each plate. Multiplication of the number by the dilution resulted in a number of cfu per sample volume. Similar counts were performed for growth on BCYE and GVPC.

The number of *L. pneumophila* in the sample were determined by the observation of the colonies on BCYE and GVPC media. Those colonies showing the characteristic ground glass appearance of *L. pneumophila* were simultaneously subcultured on to BCYE and BCYE without cysteine. Organisms were presumptively identified as legionellae if they were unable to grow in the absence of cysteine but capable of growth on BCYE.

The highest number of cfu/ml on either BCYE or GVPC was recorded as the number of *L. pneumophila* in the sample.

Observation of the colony types on each of the growth media enabled population profiles of the samples to be completed. Plates containing 30 - 200 cfu were selected where colonies of differing morphologies could be distinguished. The colony types were described using the terms of Parker & Duerden (1990) and the number of colonies of each type were counted. The organism was subcultured for identification.

2.4 IDENTIFICATION OF MICROORGANISMS FOR POPULATION PROFILES

2.4.1 Selection and subculture of bacteria for identification

The bacterial colony chosen for identification was subcultured onto R3A agar (Reasoner & Geldrich, 1985) from the R2A medium to allow more rapid growth for identification, or if isolated from BCYE or GVPC restreaked onto BCYE. The isolated organism was stained using Huckers' method for Gram's staining (Cowen, 1974) in order to ensure that only gram negative organisms were put into the Gram negative test strips for the Biolog or API.

2.4.2 Identification using Biolog

To ensure purity selected colonies were subcultured three successive times, streaked onto R3A agar, and incubated at 30°C for between 12-18 hours. The cells were removed from the agar plates using cotton swabs and resuspended into 20ml Pages' modification of Neff's amoebal saline (Page, 1967) at a density between the two standards provided with the Biolog plates at 290nm. The standards gave an optical

density (OD) of 0.35-0.40 using a 1ml cuvette with Page's amoebal saline in the blank. For slow growing organisms several agar plates were inoculated to obtain sufficient cells. 135µl of the cell suspension were inoculated into each of the wells in the 96 well Biolog microtitre plate and the plates were then incubated at 30°C. Results were recorded after 4h and 24h.

Well growth was determined using a Metertech Microplate reader model 5960 with a built in printer. The wavelength of the filter was 600nm with a source of 6v/10w tungsten halogen lamp. The microplate reader automatically deducted the OD of the control well (which contained basal media only) from the OD of each of the wells containing substrates. Results obtained from the plate reader were inserted into the Biolog data base in order to obtain an identification by matching the organisms profile with that of the Biolog data base.

2.4.3 Identification using API 20NE

API 20 NE test kits were used to aid identification of non-enteric Gram-negative rods. The culture under test was subcultured onto R3A medium and incubated for 24 - 48 h. Growth from the plates was used for the oxidation test and to inoculate the test strip following the manufactures instructions. Strips were incubated for 24 h and then results recorded. Identification of the organism was achieved by use of the API analytical profile database.

2.5 DETECTION OF PROTOZOA

2.5.1 Culture

A bacterial suspension of either *Klebsiella aerogenes* NCTC 9528 or isolates from the

chemostat cultures were used to make bacterial lawns for amoebal growth. The bacterial cells were suspended in sterile water to an absorbance of 0.160-0.190 at 290nm using a 1 cm pathlength and sterile water as the blank. A 1.0 ml volume of the bacterial suspension was poured over non-nutrient agar (Page, 1976), cells were allowed to adhere for 10 min and excess liquid poured off. The bacteria were then killed by exposure to UV light for 15 mins. The sample of water or resuspended biofilm was streaked onto the bacterial lawn and incubated at 20, 30 or 40 °C, as required, for a minimum of 7 days.

2.5.2 Detection by light microscopy

Biofilm on the surface of glass tiles was observed directly using the light microscope for evidence of amoebae and cysts. Suspensions of biofilm from other surfaces were observed by hanging drop, incubation of samples at 30 °C for a few hours resulted in observation of greater numbers of amoebae.

2.5.3 Identification of protozoa

The protozoa observed in the biofilm and planktonic samples were tentatively identified using morphology and incubation temperatures. The amoebal species were determined using "A New Key to Freshwater and Soil Gymnamoebae" by F.C. Page (1988). Other ciliated protozoa were also tentatively identified using "A beginners guide to the collection, isolation, cultivation and identification of freshwater protozoa" by B.J. Finlay, A. Rogerson & A.J. Cowling (1988) CCAP. Rotifers were also present in the culture and these were identified using "A key to British Freshwater Planktonic Rotifera" (1978) by R.M. Pontin published by the Freshwater Biological Association. Assistance in the identification of protozoa was kindly provided by Dr. T. Rowbotham from PHLS Leeds.

2.6 STAINING METHODS

2.6.1 Immunogold labelling of *L. pneumophila*

Following removal of the tiles from the chemostat, biofilms were immediately fixed for 1h in 2% (vol/vol) formalin in PBS and then dehydrated by immersion in acetone for 15min. A monoclonal antibody specific to the lipopolysaccharide of *L. pneumophila* (Sethi/LP 45, Cogent Ltd, Edinburgh) was diluted 1/40 in PBS and a 100µl volume was placed over the biofilm. The biofilm was then incubated for a minimum 8h at 4°C. Unbound monoclonal was removed by three washes in 20ml of PBS whilst gently stirring.

A 100µl volume of 1/40 goat anti-mouse immunoglobulin G conjugated with 5nm gold particles (Biocell Research Laboratories, Cardiff) was then applied to the treated biofilm and incubated for at least 8h at 4°C. Excess of the gold conjugate was removed from the biofilm by three washes in 20ml of PBS whilst gently stirring. The gold-labelled cells were visualised after treatment with a silver enhancing kit (Biocell) until sufficient resolution was achieved, as judged by microscopy. The biofilm was washed under tap water to prevent further reaction.

L. pneumophila from the chemostat was used as a positive control for the immunolabelling procedure. A 100µl aliquot of a suspension was placed onto a glass slide and air dried. A strain of *Pseudomonas paucimobilis* from the chemostat was used as a negative control and was prepared in the same manner. Further negative controls included biofilms grown under the same conditions, and subjected to the staining protocol but without either the monoclonal antibody or the gold conjugate.

The staining procedure was identical with the exception that the volumes of antibody were reduced to 10µl. Samples were viewed using episcopic Differential Interference

Contrast microscopy as described in 2.7.2.

2.6.2 Immunofluorescent labelling of *L. pneumophila*

The immunogold staining procedure was repeated except that FITC-conjugated goat anti-mouse immunoglobulin G (Sigma Chemical Co., Poole) was used to visualise the immunolabelled cells. The FITC conjugate was diluted 1/40 in PBS for use. Cells were visualised using the episcopic microscope using the FITC filter block (section 2.7.2).

2.6.3 Alcian blue-PAS Staining of biofilm polymer

The comparison of the polymer produced by the microorganisms in the biofilm was analysed using combined alcian blue-PAS polymer staining method described by Cook (1972). Biofilms were heat fixed over a Bunsen flame and stained in alcian blue (1% alcian blue in 3% acetic acid) for 5 mins. Excess stain was removed with tap water and distilled water before the biofilm was treated with 1% periodic acid solution for 2 mins. Excess stain was removed by distilled water and followed by treatment with Schiff's reagent for 8 mins. The sample was washed in running water for 10 mins before drying and viewing using the light microscope described in 2.7.2.

2.6.4 Acridine orange staining of biofilms

Biofilms were air dried and then fixed by immersion in acetone for 1-2 min. The acridine orange stain was made up using the method of Lauer *et al.* (1981) as previously used to stain biofilms by Schofield and Locci (1985). Acridine orange powder was dissolved in sodium acetate buffer (100 ml of 1M CHCOONa.3H₂O and 90 ml of 1M HCl) and pH adjusted with HCl to 3.5 to give a concentration of 100 ng/l acridine

orange. Biofilms were immersed in the solution for 2 min, washed under tap water, dried and examined using the microscope in episcopic mode (see 7.2.2.).

2.7 MICROSCOPY OF SAMPLES

2.7.1 Analysis of biofilms using scanning electron microscopy

Biofilm were examined by scanning electron microscopy (S.E.M.). This enabled the extent of extracellular polysaccharide matrix, the localisation of biofilm development and the morphology of the constituent microorganisms, including the presence of eukaryotic organisms to be assessed.

Biofilms were gently rinsed in sterile water to remove planktonic bacteria, fixed and stained using 1% osmium tetroxide in phosphate buffer at pH 6.9 for two hours. The biofilm was dehydrated using an alcohol series (25% ethanol for 15 min, 50% ethanol for 30 min, 75% ethanol for 30 min and 100% ethanol of 1 h). Tiles were mounted on 0.5" SEM specimen stubs using a high conductivity silver paint (Acheson Colloid Company, Prince Rock, Plymouth). Specimens were then coated with a 20 nm layer of gold in an Edwards 12E6 Vacuum coating unit and then examined in a Cambridge Stereoscan S2A SEM operated at 10 kV accelerating voltage. Micrographs were recorded on Ilford FP4 Plus 35 mm film, developed in an Ilford ID 11 developer and printed on Ilford Ilfospeed multigrade 3 rapid paper using a Durst L1200 photographic enlarger.

2.7.2 Light microscopy of biofilm and planktonic samples

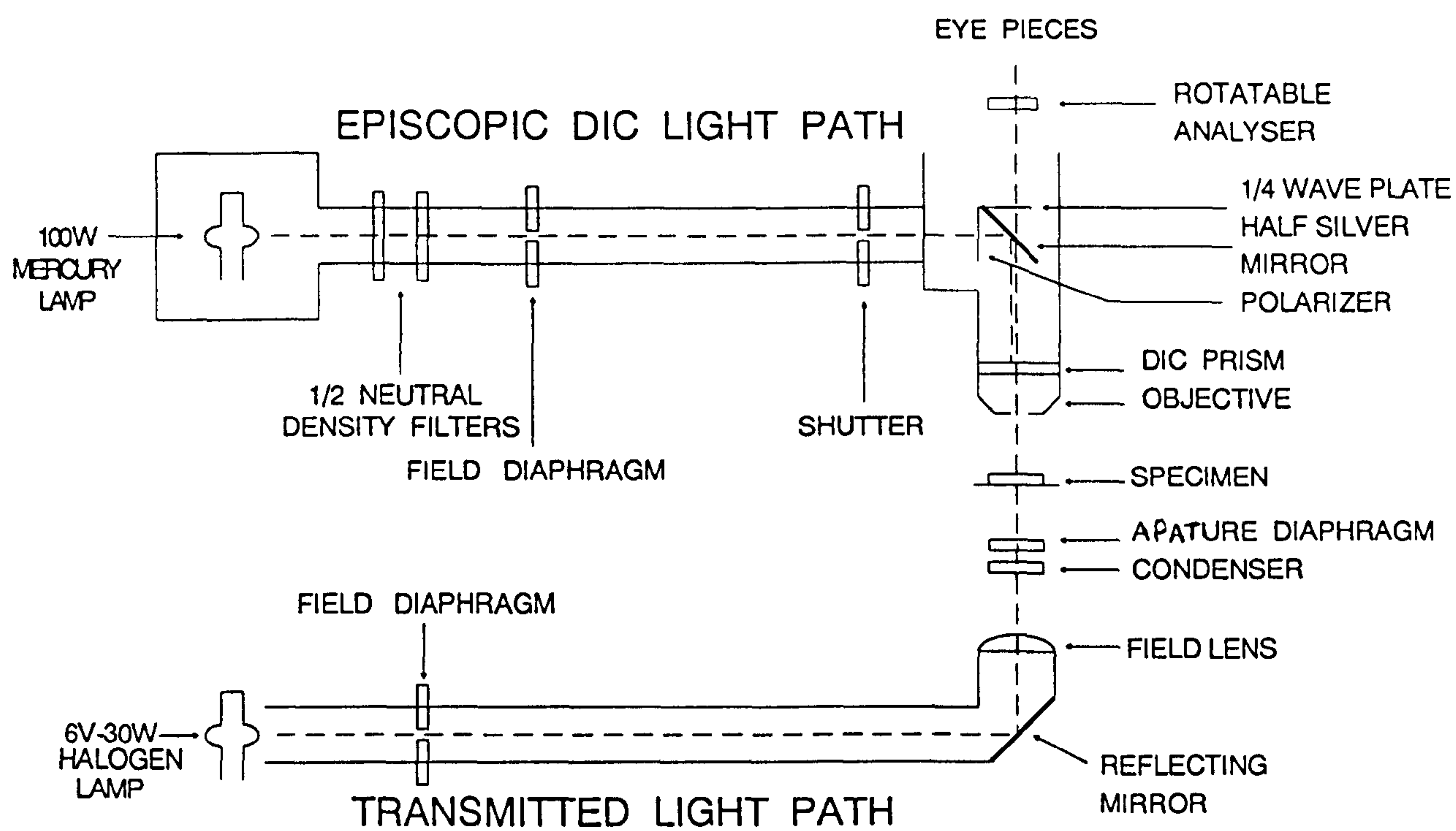
Biofilms were viewed directly under the light microscope to determine the presence and quantity of bacteria and protozoa. Samples of the planktonic microbes were also viewed by placing 20µl of culture into a 18 well slide.

The microscope used for this experimental work was a Nikon Labophot (Nikon, Tokyo) with the capacity of both transmitted light and episcopic-fluorescence microscopy using attachment EF-D (figure 3). The brightness for the episcopic light was controlled by a combination of 1/2 and 1/4 neutral density filters. For viewing fluorescein labelled samples the filter block contained an excitation filter at 450-490nm and a dichromic mirror at 510nm. For differential interference contrast microscopy (DIC) the filter block contained a polariser and a 1/4 wave plate. The immunogold staining block (IGS) contained an analyser, a 1/4 wave plate and a polariser. The microscope had an adjustable analyser fitted into the episcopic-fluorescence attachment main body.

Removable DIC prisms were also present within the objectives which were long working distance lens usually used for metallurgical purposes and consequently non cover-slip corrected. The objectives used were M Plan Apo 150/0.95 210.0, M Plan 100/0.80 ELWD 210/0 and M Plan 40/0.5 ELWD 210/0. The IGS, DIC and FITC block were interchangeable in the episcopic-fluorescence main body. The microscope was fitted with a scale on the focus adjustment knob (1 scale division was equal to 1µm) and this enabled the accurate estimation of biofilm thickness (Bakke and Olsson, 1986).

Samples were also examined using a Nikon binocular microscope at low power to determine the overall extent of colonisation.

FIGURE 2.3 : Diagram of the microscope configuration.



2.8 CHEMICAL ANALYSIS

2.8.1 Total organic carbon analysis

Total organic carbon was determined using a Beckman Model 915B Tocamaster Total Organic Carbon Computational System. For the determination of total carbon (TC) the syringe-injected liquid sample entered a combustion tube containing oxidising catalyst maintained at 950°C by microprocessor control. The sample carbon was completely oxidised to CO₂ and any water vapour was condensed and removed at ambient dew point. The resultant sample cloud was conveyed by a continuous flow of dry, carbon free carrier gas to the integral infrared analyser for the detection of the CO₂.

For the detection of inorganic carbon (IC) the sample was injected into the reaction tube containing a quartz chip wetted with phosphoric acid maintained at 155°C. Inorganic carbon reacted with the acid to liberate CO₂, which was carried via the carrier gas to the infrared analyser. Because of the relatively low temperature and the absence of the catalyst none of the organic carbon present was converted to CO₂.

The total organic carbon (TOC) was determined by the difference of the total carbon and the inorganic carbon :-

$$\text{TOC} = \text{TC} - \text{IC}$$

TC was calibrated using 100mg/l, 50mg/l and 0mg/l of sodium bicarbonate solutions. IC was calibration using 100gm/l, 50mg/l and 0mg/l of potassium biphthalate. The injection volume for both TC and IC was 50µl.

The standards and the samples were injected into the analyser in triplicate and the determination of the standard deviation and % variance ensured the accurate determination of carbon value since any samples outside a 5 % variance were repeated until a

stable analysis was achieved.

2.8.2 Densitometer analysis of biofilms

Biofilms on glass were removed from the chemostat and scanned directly without fixing or drying using a Shimadzu 500 series scanning densitometer. The beam was 0.05 x 0.5 mm in size and scanned at intervals of 0.5 mm at a wavelength of 260nm.

2.8.3 Copper concentration of chemostat culture

The determination of copper concentrations was kindly undertaken by Mr Mark Randles. Copper in the ionic form was assayed using a Philips 9100X Flame Atomic Absorption Spectrophotometer. The aqueous sample was aspirated into an air / acetylene flame and copper concentration determined by measuring the absorption at 324.8 nm.

2.9 VIRULENCE TESTING OF *L. PNEUMOPHILA*

The virulence of the strain of *L. pneumophila* serogroup 1 Pontiac isolated from the model system was assessed by Mr. S. Lever and Dr. R.B. Fitzgeorge using aerosol infection of guinea-pigs as described by Baskerville *et al.* (1981).

2.10 STATISTICAL ANALYSIS OF DATA

Colonisation of materials was compared using statistical methods. The most suitable test for this type of correlated, non-parametric data is Wilcoxon matched-pairs, signed-rank test. The power efficiency of this test relative to t-test for correlated means is reported to be 95.5% (Gibbons, 1976). Calculations were performed using "Statistics", a computer package by K. B. Smith (Blackwell Scientific Publications, 1990).

CHAPTER 3

ESTABLISHMENT OF COMPLEX MICROBIAL BIOFILMS CONTAINING *LEGIONELLA PNEUMOPHILA* ON GLASS SURFACES IN UNSUPPLEMENTED DRINKING WATER

3.1 INTRODUCTION

The aim of this work was to develop a laboratory model of a plumbing system so that the ecology and growth of pathogenic legionellae could be studied. The model system needed to reproduce the environmental conditions found within plumbing systems, so that the organisms could be studied under conditions that realistically simulated the aquatic habitat in which they occur naturally.

Legionellae are ubiquitous in the natural environment (Fliermans *et al.*, 1981) and have successfully colonised a wide range of manmade aquatic environments including plumbing systems (Dennis *et al.*, 1982), whirlpool spas (Fallon and Rowbotham, 1990) and cooling towers (Dondero *et al.*, 1980). The ability of legionellae to occupy a niche within these man-made ecosystems, despite the low nutrient availability in water systems, is the inadvertent provision of more favourable growth conditions than usually occur in the natural environment. Of the natural or manmade environments where legionellae have been detected, none require the addition of nutrient to enable the organism to grow. Legionellae are adapted to grow within water and are capable of sustaining a population by using the nutrients available in the water. With this factor in mind, filter sterilised tap water was selected as the sole source of nutrient for the model system.

The organic materials present in water are however, not directly utilisable by the legionellae, growth can only be achieved when other microorganisms are present to support its growth by providing byproducts that are metabolisable (Wadowsky *et al.*, 1988 and West *et al.*, 1989). The aquatic environment in which legionellae grow contains a complex microbial consortium. Many organisms have been shown to encourage growth of the bacterium including amoebae, ciliates, bacteria, and algae. The model system was therefore designed to sustain the growth of *L. pneumophila* by

providing the consortium of microorganisms that occur naturally with this organism.

In order to include the whole population of microorganisms present in the ecosystem in which *L. pneumophila* was growing, sludge from a calorifier implicated in an outbreak of Legionnaires' disease, was used to inoculate the model system. This inoculum contained a diverse range of microorganisms, some of which would support the growth of the *L. pneumophila* and others of which would be inhibitory to growth, but all of which would be required in order to study the interactions of the community in which *L. pneumophila* grows.

Another factor considered important in the model design was the use of a continuous supply of sterile water as the nutrient for the community. As water is removed for use from plumbing systems, fresh water is added to maintain a fixed volume. Therefore the use of a recirculating model system for the study of plumbing systems would not be appropriate. Furthermore, in some cases the accumulation of toxic products or metabolites may result in inhibition of growth (Tison, *et al.*, 1980). Therefore a continuously fed system was selected for the growth of the microbial consortium.

The above criteria for the design of the model could be best fulfilled by the use of a chemostat or continuous culture system. The use of a mixed population of microorganisms using sterile tap water as the nutrient source could be incorporated into the chemostat approach to study the ecology of aquatic systems. The conditions within the model could be closely defined, controlled and reproduced.

Many of the studies of the ecology of *L. pneumophila* have focused on the organisms in the planktonic phase. Plumbing systems are composed of two distinct but interactive components, the circulating water within the plumbing systems and the materials that comprise the pipework and fittings. Microorganisms are present in the water phase and on the surface of the materials in biofilms. Although traditional chemostat design

minimises growth on surfaces, successful growth of the *L. pneumophila* in the complex aquatic consortium would enable the insertion of tiles in to the model system to allow biofilm development to be assessed.

Biofilms were developed on the surface of glass coupons. Although glass is rarely selected as a plumbing material the development of the model using this as the material has several advantages:-

- 1) it is biologically inert
- 2) it is suitable for viewing under all types of microscope
- 3) stained biofilm could be visualised
- 4) biofilm could easily be removed from the surface and viewing of the tile would confirm successful recovery.
- 5) it provides a "control" surface for events occurring on the wall of the vessel

The initial experiments described in this chapter were concerned with development of the model system, the production of biofilm on the surface of glass and the assessment of the reproducibility of biofilm development. The study of the colonisation of glass permitted the determination of the three dimensional structure of the biofilm and the location of the *L. pneumophila* within it.

The model system was known to contain virulent *L. pneumophila* at the outset of the experimental work. In order for the model to be valid, the strain of *L. pneumophila* should not lose virulence. After the experimental work was completed, the *L. pneumophila* was isolated from the chemostat model, and virulence reassessed using the guinea pig model of infection.

3.2 MATERIALS AND METHODS

3.2.1 Development of the model

After inoculation of the vessels, the bacterial populations were allowed to grow in batch culture for 3 days. Medium was then continually added and the planktonic populations were monitored regularly and the system was allowed to reach steady-state (usually 3 weeks). If population diversity was reduced or the population of *L. pneumophila* declined during the three weeks, the dilution rate was reduced. The system was reinoculated and the stabilisation period repeated.

3.2.2 Evaluation of bacterial identification by API and BIOLOG

The efficacy of the identification systems was compared using NCTC reference cultures chosen because they were derived from water sources and represented species commonly isolated from water (see over page). The ampoules were opened, the organisms cultured onto nutrient agar and then subcultured onto R3A medium. After ensuring bacterial purity, the bacterial isolates were inoculated into the Biolog and the API identification systems so that identifications could be compared as described in section 2.4. The Biolog system is designed to be extendible so species not present within the data base could be assigned a profile that could be useful for further identifications. The API system is a more closely controlled data base and the instructions state that species not contained within the database are unsuitable for identification using the API system, for this reason some species were not included in the comparison since they were not included in the API 20 NE database.

The strains tested in the Biolog and API systems included :-

NCTC 8698 <i>Acinetobacter calcoaceticus</i>	NCTC 8251 <i>Aerococcus viridans</i>
NCTC 10362 <i>Aeromonas formicans</i>	NCTC 8049 <i>Aeromonas hydrophila</i>
NCTC 11215 <i>Aeromonas sobria</i>	NCTC 12119 <i>Buttiauxella agrestis</i>
NCTC 7420 <i>Corynebacterium spp.</i>	NCTC 11409 <i>Flavobacterium rubrum</i>
NCTC 10797 <i>Flavobacterium breve</i>	NCTC 12158 <i>Klesiella planticola</i>
NCTC 12146 <i>Klebsiella terrigena</i>	NCTC 9173 <i>Klebsiella</i> sp. type 53
NCTC 9527 <i>Klebsiella aerogenes</i>	NCTC 9528 <i>Klebsiella aerogenes</i>
NCTC 11395 <i>Pseudomonas acidovorans</i>	NCTC 10367 <i>Pseudomonas alcaligenes</i>
NCTC 10038 <i>Pseudomonas fluorescens</i>	NCTC 10898 <i>Pseudomonas mendocina</i>
NCTC 10499 <i>Pseudomonas maltophilia</i>	NCTC 11030 <i>Pseudomonas paucimobilis</i>
NCTC 10698 <i>Pseudomonas testosteroni</i>	NCTC 11167 <i>Pseudomonas vesicularis</i>
NCTC 10994 <i>Pseudomonas coprophilus</i>	NCTC 10807 <i>Pseudomoas xylosoxidans</i>

3.2.3 Biofilm development on glass

The chemostat model was set up as described in 2.1 using Thames water as the sole nutrient source. The second vessel was used for the production of biofilms with a dilution rate of 0.2 h. The temperature was maintained at 30 °C with the oxygen at 20 % DOT. Glass tiles were prepared as described in section 2.2, inserted into the chemostat at day 0 and biofilm allowed to develop on the surface of the tiles. Biofilm generated on the surface of the tiles were removed for culture after 1, 4, 7, 14, 21 and 28 days.

3.2.4 Assessment of the reproducibility of biofilm formation

Although attempts were made to ensure all biofilms were developed and recovered under the same conditions, variations would inevitably occur. The most likely sources of variation could occur during biofilm development and during the scraping off process. The difference in enumeration in liquid samples was minimal suggesting that the variation of the sample plating following serial dilution was small (Section 3.3.3). Although errors could not be attributed to any particular factor, the overall accumulative effects could be measured by comparing the recovery of bacteria from tiles simultaneously immersed into the chemostat model.

A total of 16 glass tiles were simultaneously inserted into the chemostat second vessel at 30 °C with Thames water as the nutrient source. Biofilms were allowed to develop for 32 days, the glass tiles were then removed and the numbers of bacteria determined as described in Section 2.3.

3.2.5 Analysis of the three dimensional structure of biofilms

Glass tiles were inserted into the second vessel under the conditions described in Section 2.2.1 and 2.2.2. Tiles were removed from the model system after 28 days and immediately placed in the scanning densitometer as described in Section 2.8.2. Tiles were also examined under the binocular microscope which enabled biofilm depth and structure to be recorded.

3.2.6 Immunolabelling of *L. pneumophila* in biofilms

Biofilms were developed on glass tiles for 7 days at 40°C and immunolabelled as described in 2.6.

3.2.7 Assessment of the virulence of *L. pneumophila* from the model.

The strain of *L. pneumophila* serogroup 1 Pontiac inoculated into the chemostat had been responsible for several cases of Legionnaires' disease. It was not known if the virulence had been maintained within the chemostat model. After the conclusion of the three years practical work the virulence of the strain of *L. pneumophila* held in the model was tested to determine whether infectivity could be maintained over time. The *L. pneumophila* serogroup 1 was isolated from the chemostat culture at 40 °C and subcultured only once prior to virulence testing.

The virulence testing was kindly carried out by Mr. S. Lever and Dr. R.B. Fitzgeorge at PHLS CAMR using the aerosol infection method of virulence testing (Baskerville *et al.*, 1981). Three batches of five guinea pigs were infected with inocula containing 1×10^{10} , 1×10^9 and 1×10^8 cfu *L. pneumophila* respectfully using a Henderson-type apparatus. The retained inhaled dose of bacterium was assessed using one animal and the remaining four animals were monitored for infection.

3.3 RESULTS

3.3.1 Establishment of the chemostat model

The chemostat microbial population reached steady state after an initial exponential growth phase and *L. pneumophila* represented 1% of the total bacterial flora at 30 °C. The population contained a diverse range of microorganisms, principally gram negative bacteria. The flora included species of *Alcaligenes*, *Acinetobacterium*, *Aeromonas*, *Chromobacterium*, *Flavobacterium*, *Methylobacter*, *Pseudomonas* and actinomycetes.

3.3.2 Identification of the bacterial population

The results of successive incubations of the NCTC strains in the Biolog system of bacterial identification of microorganisms were compared. The large numbers of variable results obtained using the same NCTC strain was an indication that the acceptable range of OD required was too narrow to ensure that all positive results were recorded. There were several problems associated with the use of the new database, several genera were not included at the onset of the work, and updating of the database led to organisms being assigned to different species or even genera. If insufficient growth was achieved within the 24 h period suggested by the manufacturers, the strain being tested was misidentified as *Moraxella bovis*, extending incubation times often led to alternative identifications. This often produced a diverse range of identifications for the same organism. For example, a known strain of *Bordetella bronchiseptica* was incubated for 4 h and produced an excellent identification for *Philomiragia bacterium* with a similarity of match of 0.938 and a distance (or goodness of match) of 0.304. This identification was maintained after 24 h but a further 8 h incubation led to identification of the species as *Bordetella bronchiseptica*, the identification was described as good, with a similarity of 0.754 and a distance of 2.304. This problem of insufficient

incubation length in some species was mirrored by too rapid growth by other species. *Klebsella* species often produced positive wells over the whole plate within 8 h, even if these were read after 4 h growth was too extensive to determine species in many cases. The problem of growth rate was intrinsic to the database since the manufactures recommended time intervals for measurement but did not specify the growth temperature. It is likely that different organisms were grown at different temperatures in order to develop the database and consequently any standard method of treatment of unknown species will lead to variable results dependent on incubation temperature.

The API system of bacterial identification was found to accurately identify organisms present within its database. The database was developed for clinical use and contained profiles of a limited number of environmental species. Several species gave clear positive and negative results but produced unacceptable profiles or no identifications because the database was not sufficiently comprehensive. Another common fault was the failure of the more slow growing environmental isolates to achieve sufficient growth in the API strip to attain colour changes or density differences in the time allocated for valid results to be obtained. Results obtained after the specified 48 h are considered unreliable and many of the environmental isolates did not produce sufficient growth until after this period.

The Biolog system was capable of producing data for almost all of the isolates inoculated into the wells since colour production is due to reduction of tetrazolium violet and not an indirect pH shift. However, the closer stringency of inoculating procedures to follow updated manufacturers guidelines still resulted in differing profiles for the same strains.

Identification of the microorganisms present in the cultures was achieved by a combination of both identification systems, this resulted in identification to genus level. The only organisms that could be reliably identified to species level using either the API

or Biolog systems were the pseudomonads.

3.3.3 Biofilm development on glass surfaces

The development of biofilm

Glass surfaces were rapidly colonised by the population of microorganisms in the chemostat and after only 24 h there was a total bacterial population of 1.00×10^4 cfu cm^{-2} . The *L. pneumophila* were present in the biofilm at 1.00×10^2 cfu cm^{-2} , accounting for 1 % of the total flora. After 4 days the numbers of legionellae increased to 3.00×10^2 cfu cm^{-2} . As the biofilm on the glass surfaces matured the total number of non-legionellae increased to a maximum of 1.50×10^5 cfu cm^{-2} by 14 days. The legionellae numbers reached a maximum of 1.60×10^3 cfu cm^{-2} after 21 days and remained at approximately 1 % of the biofilm flora.

Morphology of the biofilm

The biofilms were examined using the scanning electron microscope. After 24 h the surface of the glass tile supported a monolayer of bacterial cells which were spread thinly over the surface leaving large areas uncolonised. The bacterial morphology indicated that the majority of organisms were rod shaped and occurred either singly or growing in chains. The presence of *Hypomicrobium* spp. was indicated by the morphology of stalked bacterium in the biofilm.

After 7 days the bacterial flora had developed into biofilm in small localised areas and the cells had become obliterated by a layer of extracellular material, possibly biofilm polymer. The surface supported a diverse range of microorganisms spread over the exposed glass tile with some areas not containing bacteria.

By 21 days most of the glass tile was covered with biofilm. Cells could be

Figure 3.1 The colonisation of glass surfaces by total flora (■) and *L. pneumophila* (●) in Thames water at 30 °C

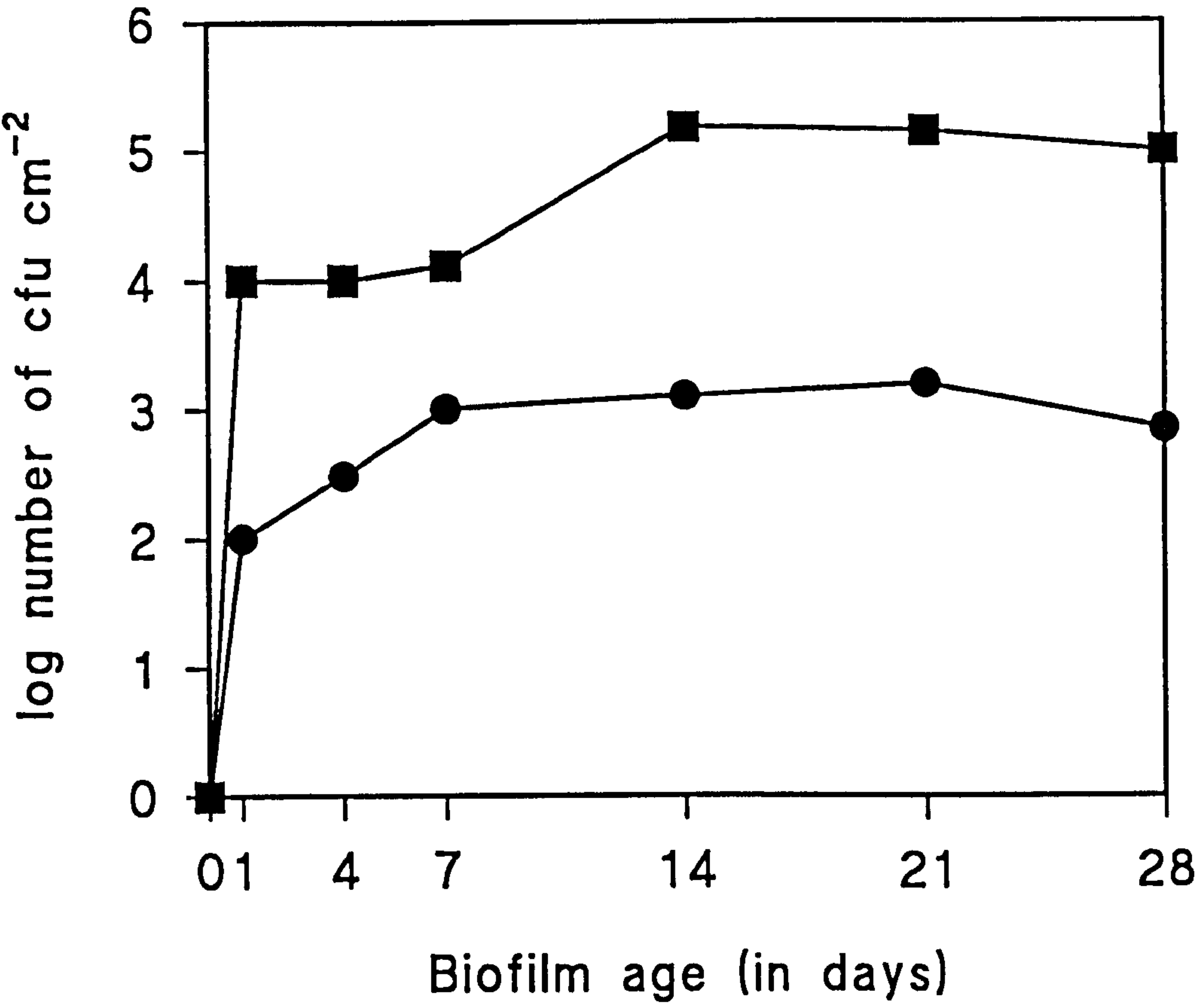
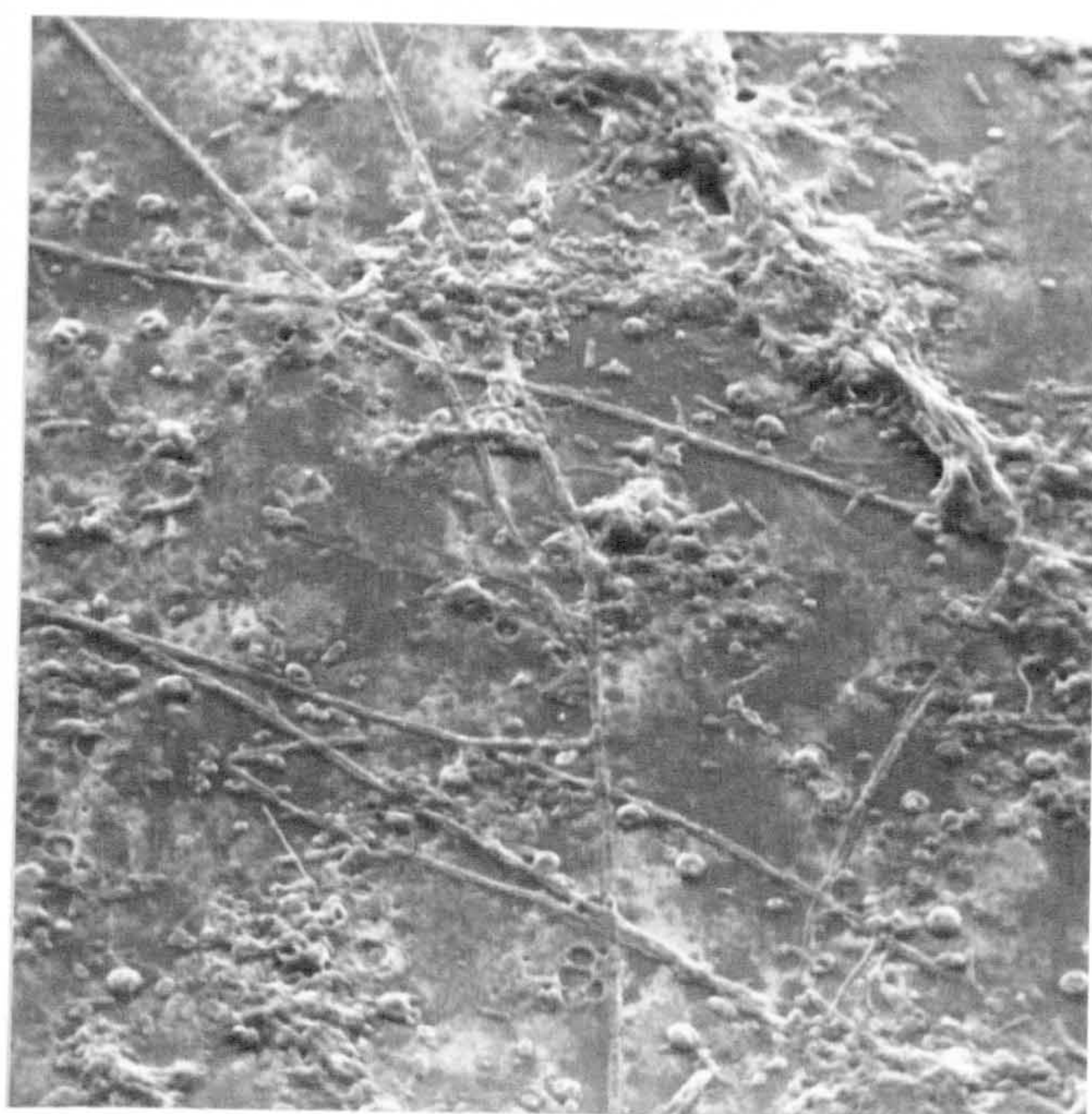
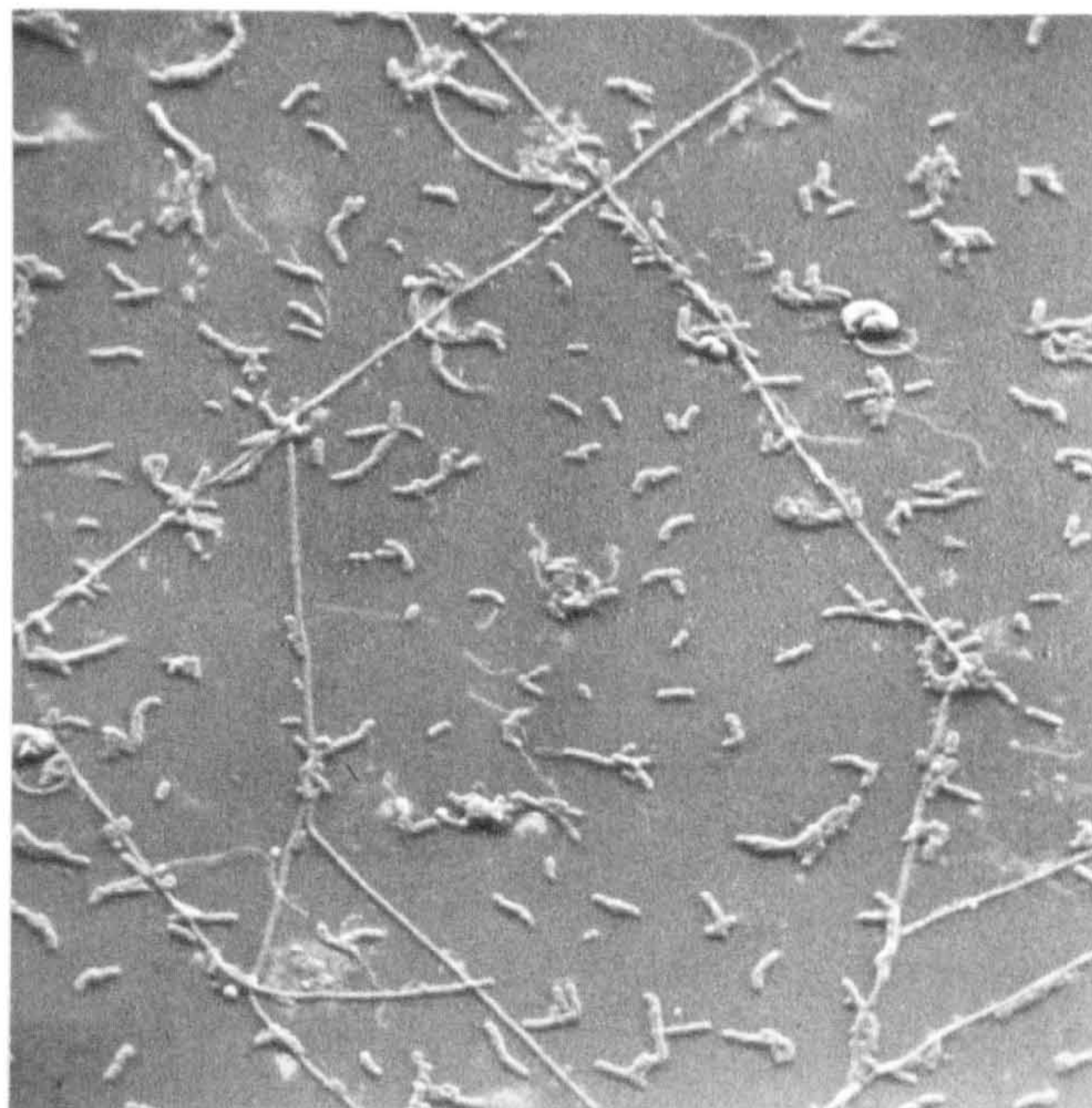


Figure 3.2 Scanning electron micrographs of biofilms developing on glass surfaces after 1, 7 and 21 days.



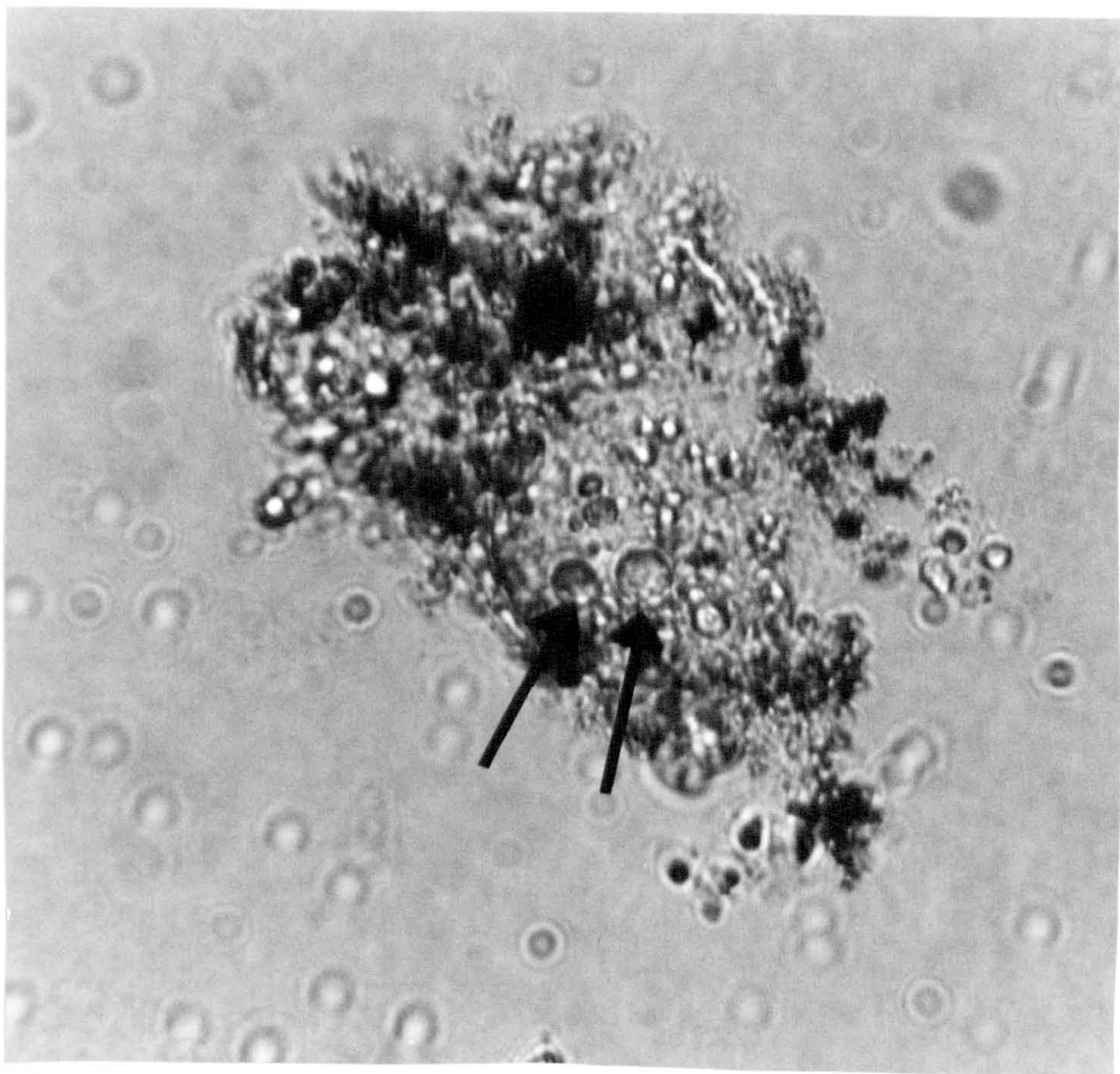
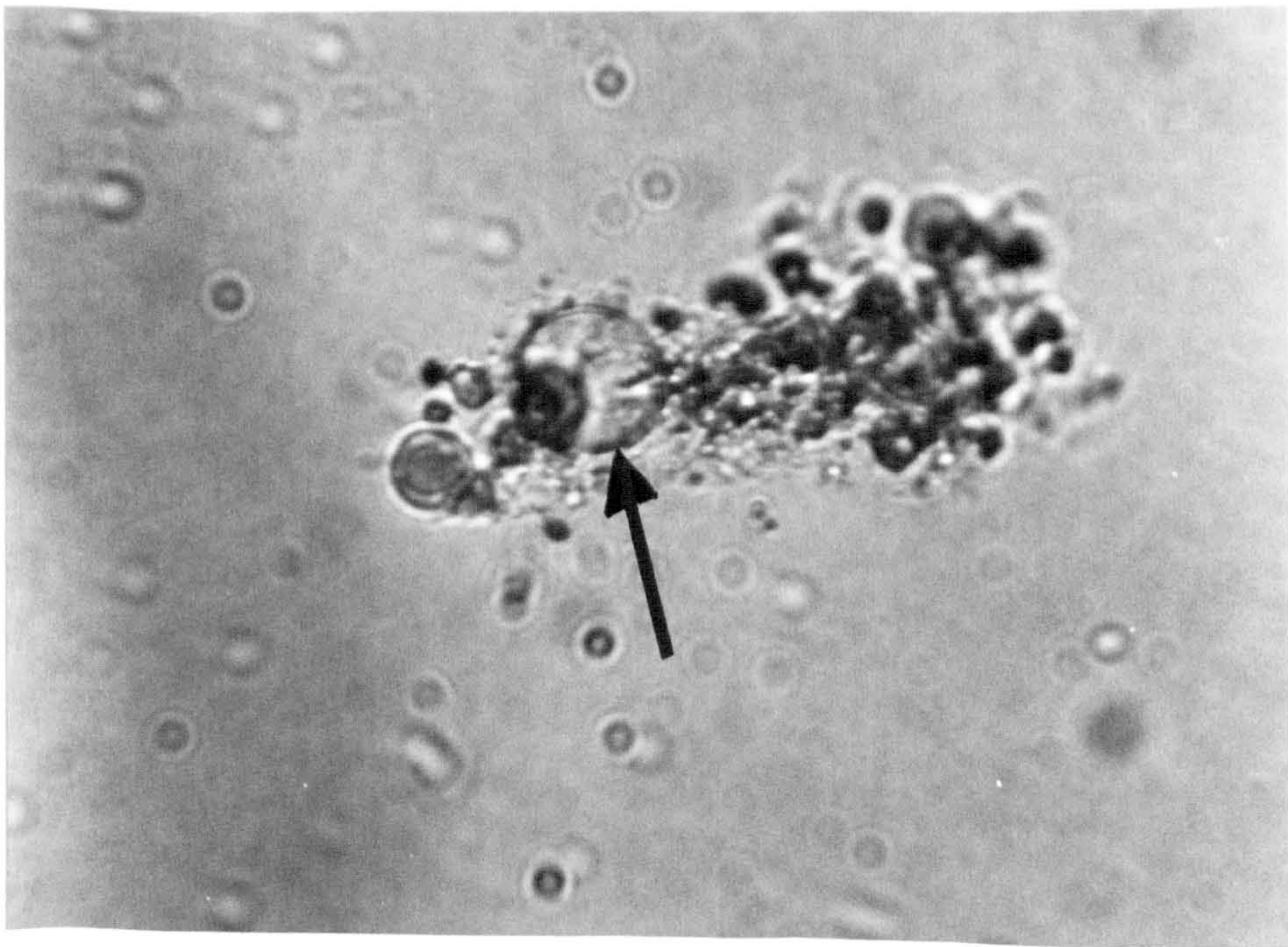
distinguished within clumps and on the surface of the glass. Some areas of the glass tile were covered by thick layers of biofilm which contained large amounts of polymer.

Amoebae and other protozoa in the biofilms on glass

Amoebae were commonly observed on the glass biofilms at 30 °C mostly occurring as cysts (Figure 3.3). Amoebae isolated from the culture included *Hartmannella vermiformis*, *Hartmannella cantabrigiensis* and *Verillifera bacillipedes*. Also present within the culture were an unknown testate amoeba, a tiny colourless euglenoid biflagellate and rotifers. *Hartmannella vermiformis* is a common limax amoeba which feeds on bacteria and readily forms cysts. The amoeba is capable of anaerobic growth and can support the growth of *L. pneumophila* (Wadowsky *et al.*, 1988). *Hartmannella cantabrigiensis* is a fairly common, rapidly growing limax amoeba. This amoeba feeds on live bacteria and cannot grow anaerobically. *Hartmannella vermiformis* did not appear to support the growth of the *L. pneumophila* serogroup 1 Pontiac, since the numbers of the bacteria declined in coculture. The unknown testate amoeba was slow growing at 30 °C and found in association with *Hartmannella vermiformis* cysts. The tiny colourless euglenoid biflagellate appeared to absorb nutrient through the cell wall.

Both resting stages and motile rotifers were also present on the surface of the glass tiles. The species were identified as *Rotari neptunia*. Other protozoa included the predatory *Lacrymaria* spp. which are known to ingest amoeba.

Figure 3.3 Micrographs of amoebal cysts (indicated by arrows) within biofilms on glass surfaces.



Bacteria colonising the glass surfaces

The most abundant species were the pseudomonads which accounted for at least 70 % of all biofilm flora developing on glass. The pseudomonads present in the chemostat included *P. aeruginosa*, *P. acidovorans*, *P. corprophila*, *P. diminuta*, *P. fluorescens*, *P. mendocina*, *P. paucimobilis*, *P. stutzeri*, *P. testosteroni*, *P. vesicularis* and *P. xylesoxidans*. The other bacteria included other Gram negative bacteria including *Acinetobacter spp.*, *Aeromonas spp.*, *Alcaligenes spp.*, *Flavobacterium spp.*, *Methylobacterium spp.*, *Klebsiella spp.* and *Vibrio spp.* The only Gram positive organisms were actinomycetes and *Aspergillus fumigata* which were also present in low numbers.

Legionella pneumophila serogroups 1, 4, 6 and 10 were present in the chemostat model and of these *L. pneumophila* serogroup 1 Pontiac was predominant.

3.3.4 Reproducibility of colonisation

The distribution of the total numbers of cfu cm⁻² in the colonisation of glass at 30 °C in Thames water are shown in figure 3.4. The total biofilm flora on the 32 day old tile ranges from 1.4 to 2.2x10⁵ cfu cm⁻². The population follows a Poisson distribution and the mode is 1.9x10⁵ cfu cm⁻². The range of 0.9 of a log unit represents a 10 % margin of error. The mean number of total flora after 32 days was 1.84x10⁵ cfu cm⁻².

The *L. pneumophila* also had a Poisson distribution over the samples of biofilm with a distribution from 1.4 to 2.3 x 10³ cfu cm⁻² (figure 3.5). The population had a mode of 1.7x10³ cfu cm⁻². The range was 0.1 of a log unit which represented an error of 10 %. The mean number of *L. pneumophila* was 1.72x10³ cfu cm⁻².

Biofilms recovered at 30°C in Thames water have been useful in determining the limitations of the chemostat model, this experiment has shown that the variation in the biofilm formation in the chemostat is sufficiently small to enable the model to be utilised for the comparison of material colonisation or biocide testing since reproducibility levels are acceptable.

Figure 3.4 Distribution of total flora from biofilm samples developed at 30 °C in Thames water after 32 days.

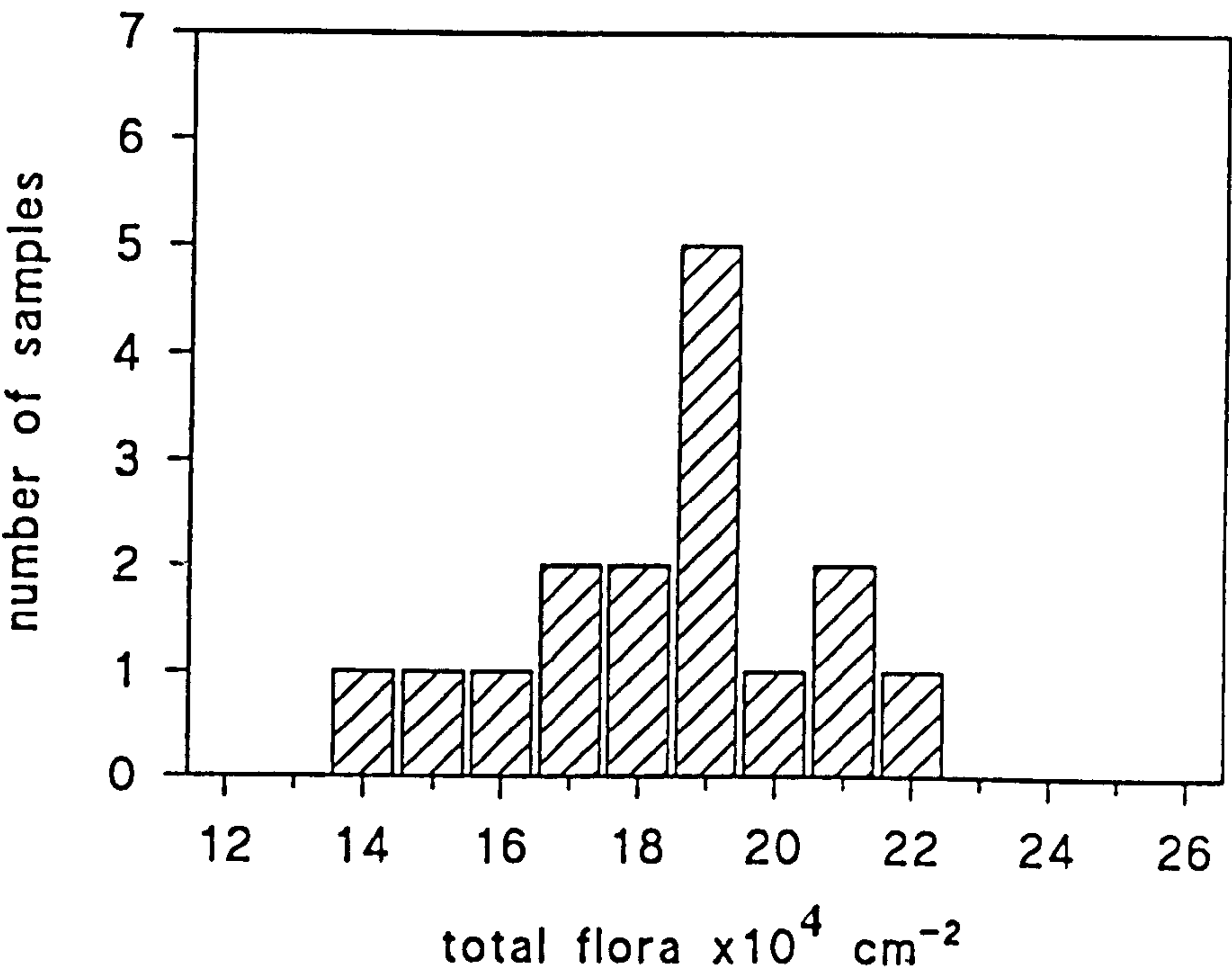
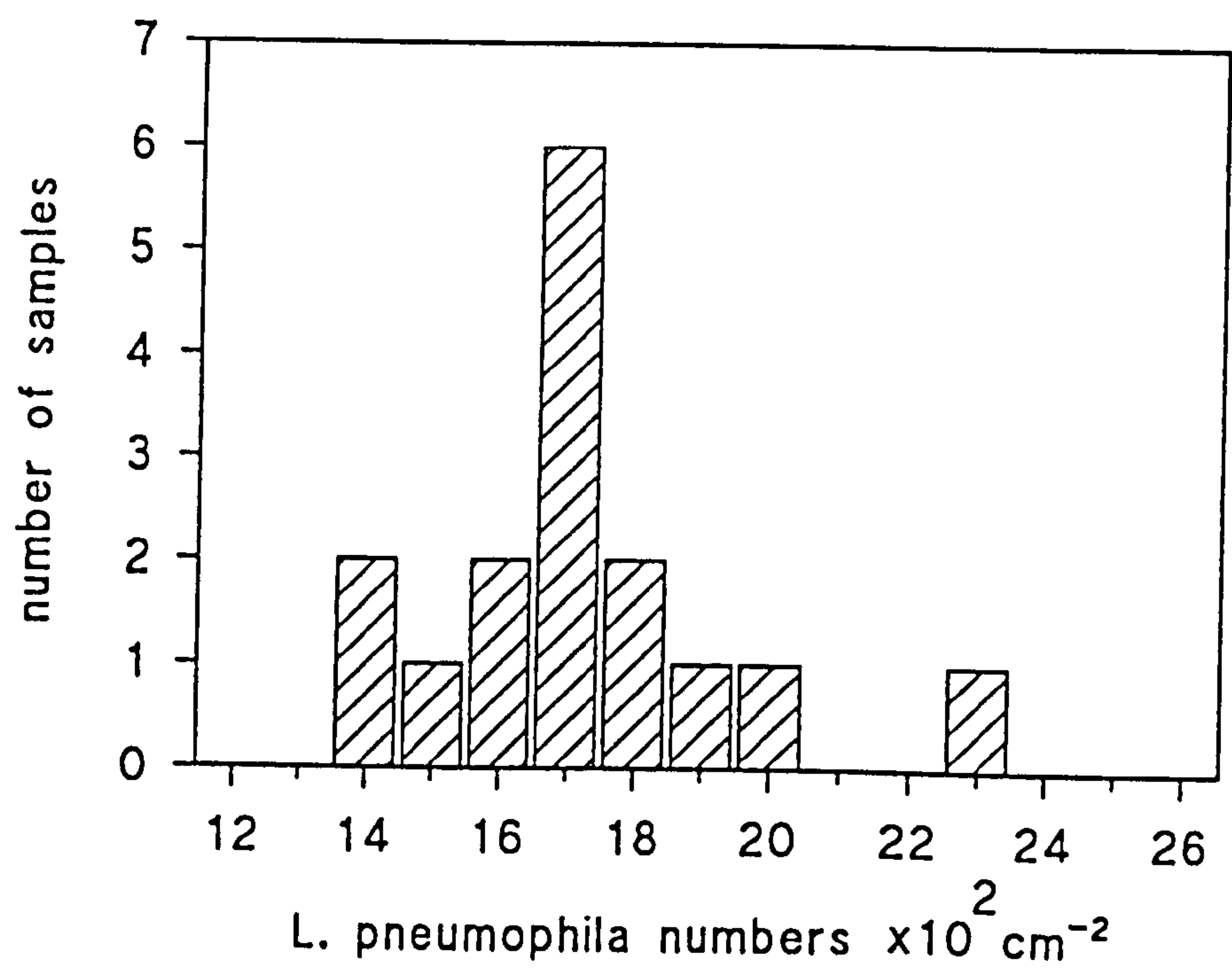


Figure 3.5 Distribution of *L. pneumophila* in biofilms developed at 30 °C in Thames water after 32 days.



3.3.5 Three dimensional structure of biofilms

Direct visualisation of unstained biofilms

When biofilms were viewed directly without staining using the Nikon binocular microscope, it was apparent that the whole surface of the glass tile was not colonised by microbes, but some areas had no colonisation (Figure 3.6a). The areas where bacteria were present consisted of low basal layers of biofilm with tall stacks of biofilm coming up from the surface. The basal layer was 5 μm in height and the stacks of microorganisms reached up to 100 μm . The absence of colonisation between these stacks enabled flow of water and movement of protozoa within channels.

The microorganisms occurring in the basal layer and in the stacks appeared to exist within microcolonies as similar coloured microorganisms could be observed in discrete clusters (Figure 3.6b). The bacteria which grew on low nutrient R2A plates had similar colours to those occurring in the biofilm, suggesting pigment production in the environment was occurring and this was species dependent.

The biofilm specimen was examined spectrophotometrically using a scanning densitometre as described in 2.8.2. The cross section of the biofilm showed that the optical density varied between 0.15 and 1.30 at 260 nm (Figure 3.7). This observation suggests that the biofilm varied considerably in thickness across the surface of the glass tile and supports the conclusions obtained from light microscopy.

Use of acridine orange to view biofilm

The biofilm could be visualised using acridine orange but the stain tended to colour the

surface and any non biological debris as well as the bacteria in the biofilm. The need to stain the biofilms and the lack of ability to enlarge made this technique less useful than other techniques and a combination of SEM and DIC was used for further work.

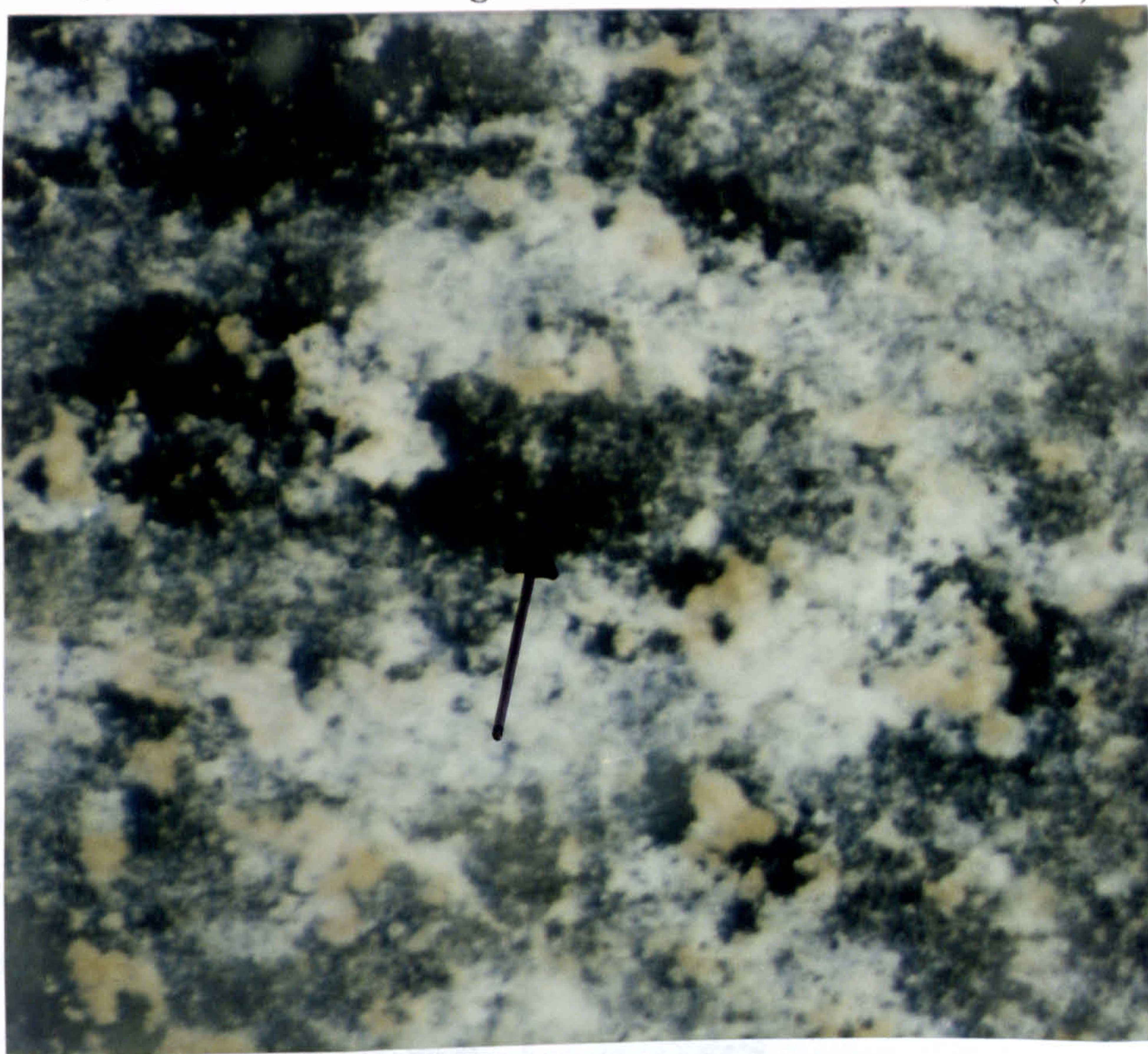
Use of DIC to view biofilms

The combination of episcopic light DIC microscopy (Section 2.7.2) and non coverslip corrected, extra long working distance lenses allowed visualisation of biofilm on opaque surfaces at up to 1500x magnification at the eye pieces. As the tiles were curved, conventional lenses would not focus using conventional incident light. The use of episcopic DIC was most successful when the biofilm was viewed directly without oil or coverslip, these interfaces reflected light from their surfaces so interfering with the light returning to the specimen and preventing specimen visualisation. A particular attribute of the DIC microscopy is that the method allowed clear visualisation of cells within the biofilm without the need of any staining procedure.

Plastic and glass surfaces showed colonisation similar to that observed under the binocular microscope. The surfaces were rapidly colonised and appeared to form biofilm which was initially localised zones of cells with large areas uncolonised. As the biofilm matured the clusters of cells increased in height and the bacterial growth infilled the previously uncolonised zones. The basal layer of cells was 5 μm in height with tall stacks of bacterial microcolonies reaching 100 μm .

Figure 3.6 Micrographs of unstained biofilm showing that some areas were uncolonised (a) and that the microorganisms occurred in microcolonies (b).

(a)



(b)

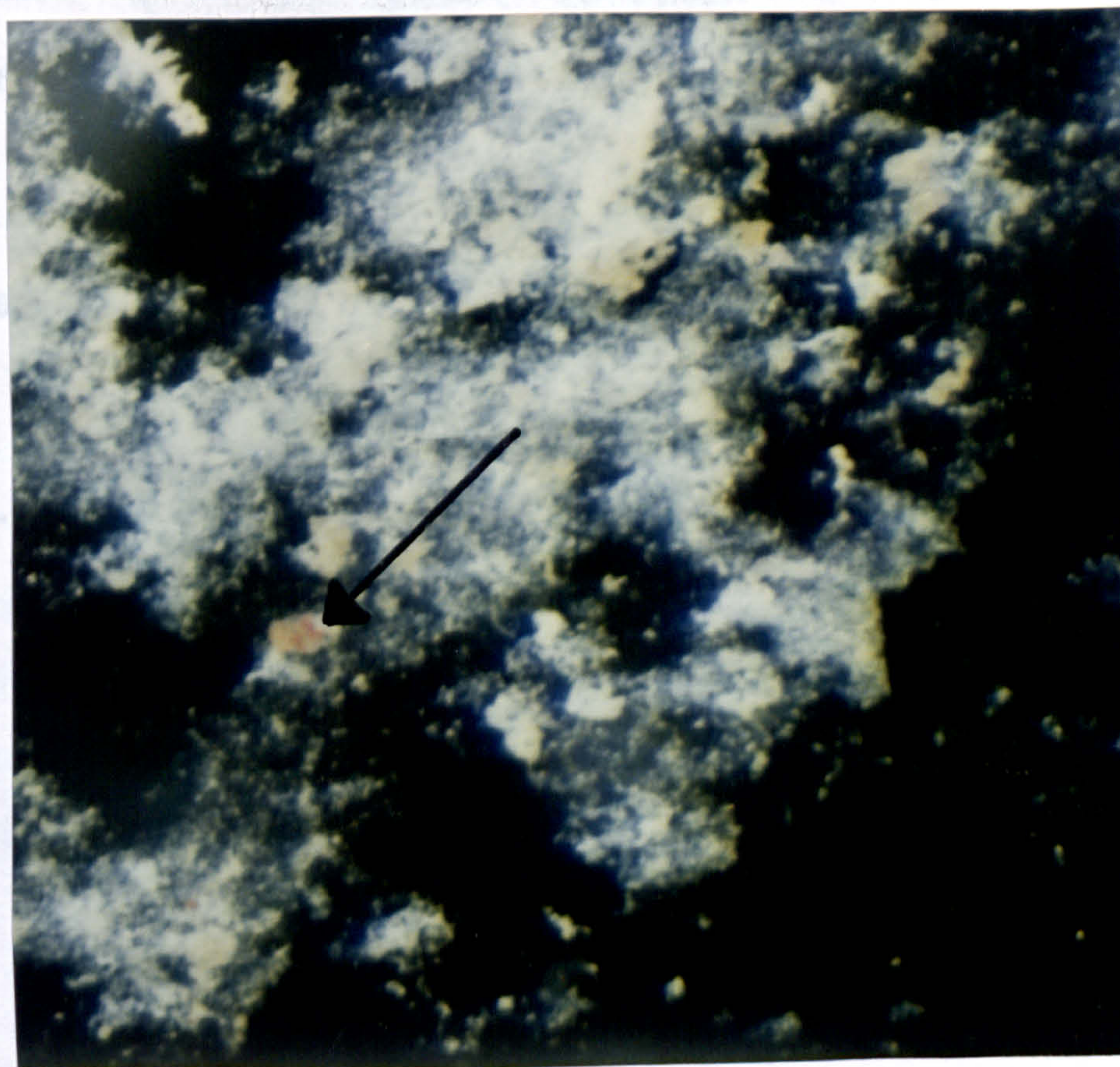
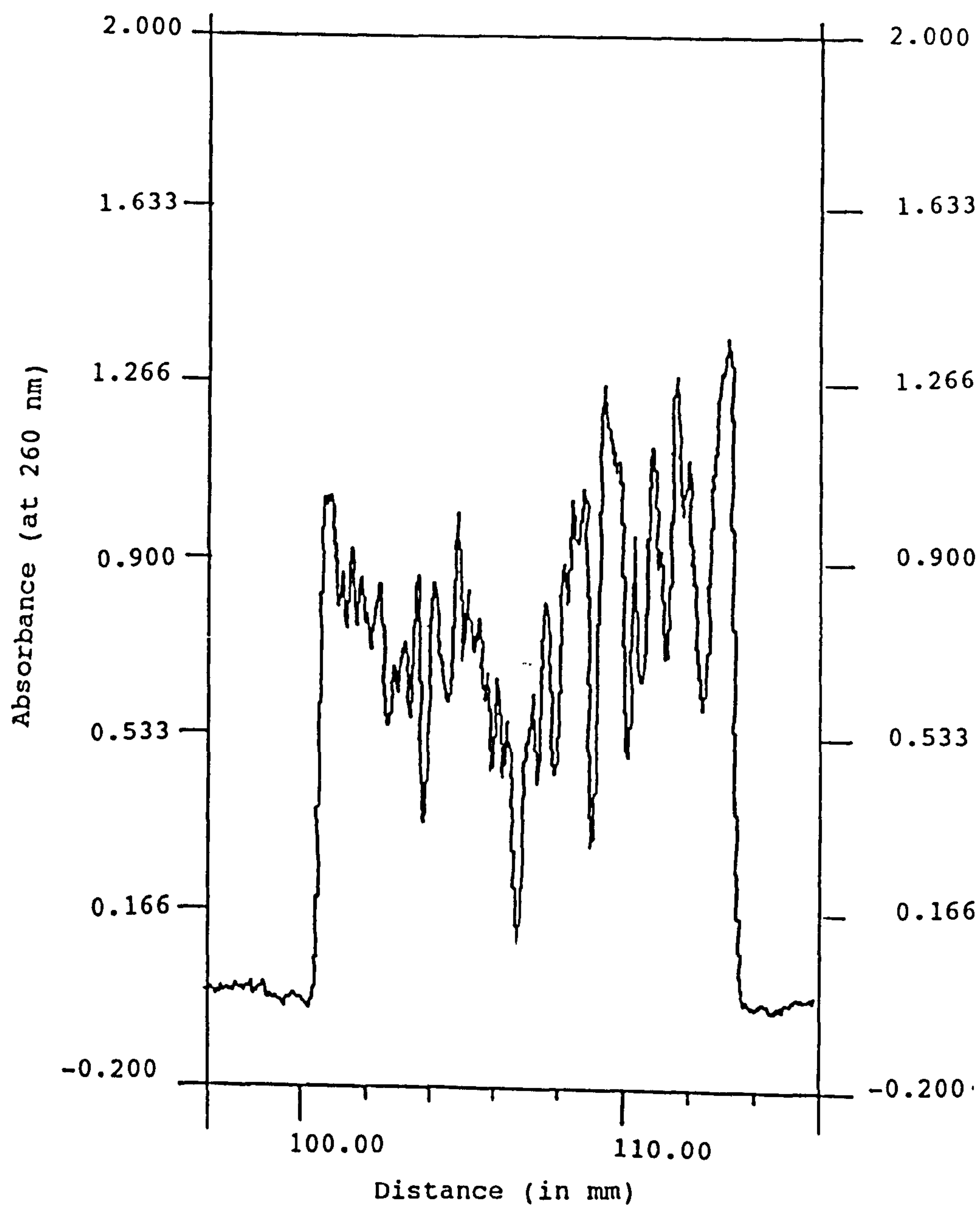


Figure 3.7 Scanning spectrophotometer cross section of a 21 day biofilm on glass



0.05 x 0.5 mm beam scanning every 0.5 mm.

3.3.6 Immunolabelling of *L. pneumophila* in biofilms

Immunogold labelling

A pure culture of *L. pneumophila* from the chemostat model that was used as a positive control was clearly labelled using the immunogold staining procedure, and there was a minimum of nonspecific, background staining. The negative controls of biofilms containing legionellae incubated in the absence of either the monoclonal antibody or the gold conjugate were all unlabelled by the gold. Similarly, a pure culture of *P. paucimobilis* was also unlabelled.

Observation of biofilm treated with immunogold indicated that the legionellae in the biofilm were also successfully labelled with gold particles. Microscopic observation of the immunolabelled biofilm showed that, although the fixing procedure resulted in the dehydration and compression of the biofilm, there was no appreciable loss of biofilm or apparent loss of bacterial cells. The legionellae appeared as short rods on the biofilms developed in tap water; the labelled pure cultures contained both short rods and much longer, pleomorphic rods. The legionellae were found dispersed singly over the biofilm but were more commonly observed in distinct groups and microcolonies (Figure 3.8).

There was little evidence of nonspecific binding of the monoclonal antibodies or the gold particles, despite theoretical concern over the sequestering properties of the biofilm matrix. The appearance of the immunogold-labelled bacteria was similar when they were viewed with either the DIC filter or the immunogold staining filter. The DIC block afforded the advantage of adjustment of the analyser, which improved contrast between the gold-labelled legionellae and the unlabelled biofilm bacteria. The absence of legionellae in biofilms generated at 50 °C was confirmed by the absence of gold labelled cells and provided an additional negative control.

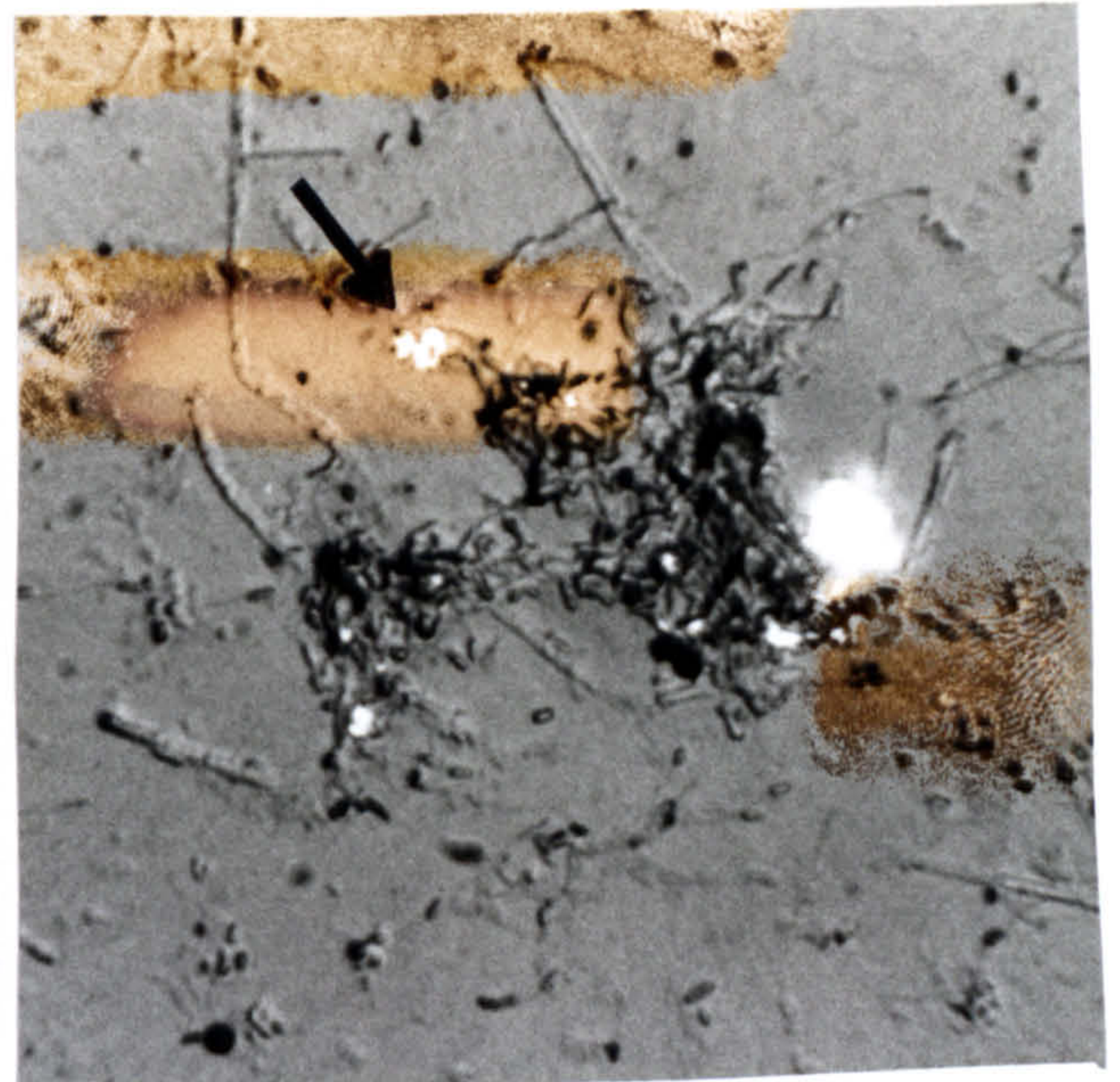
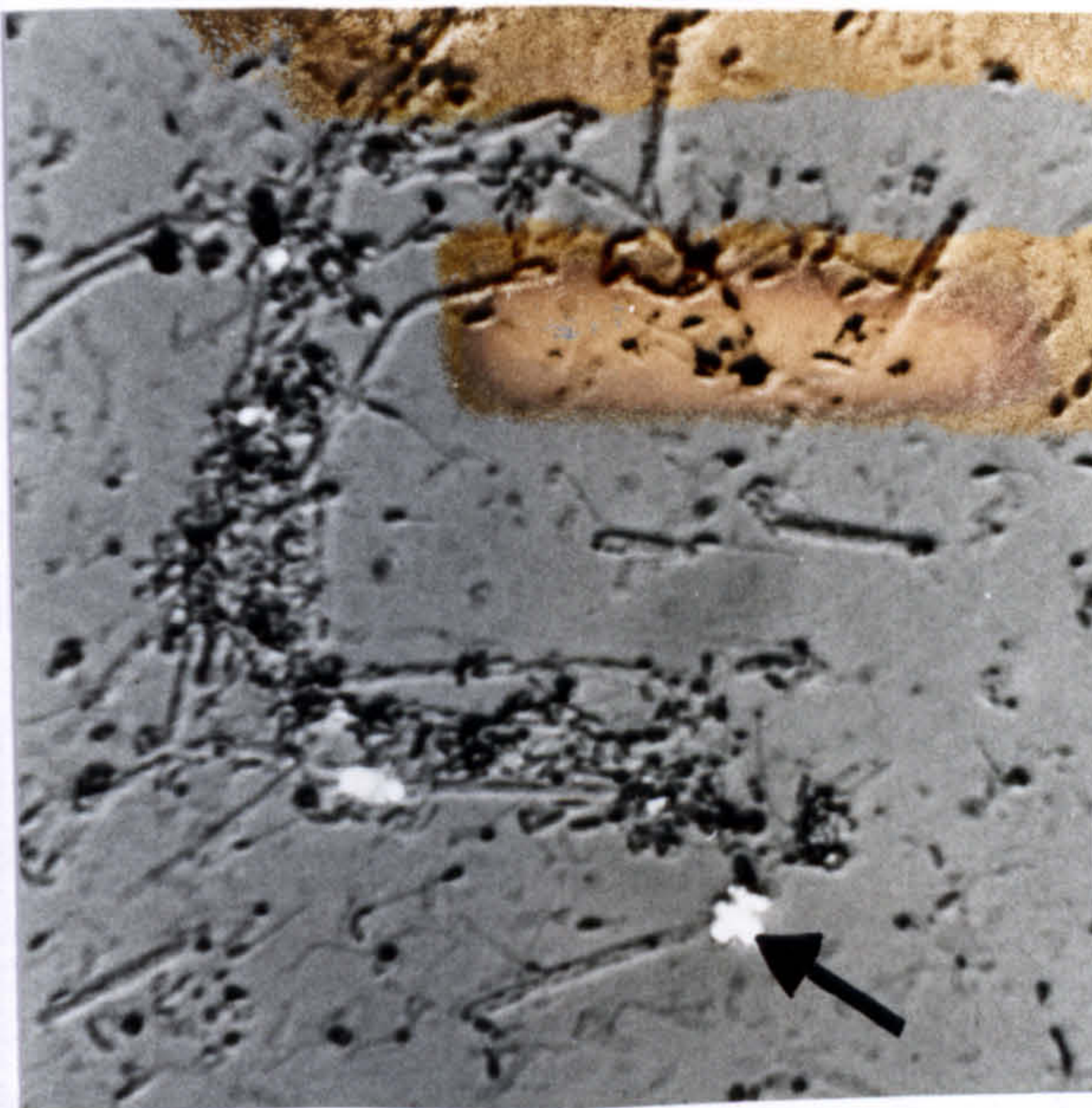
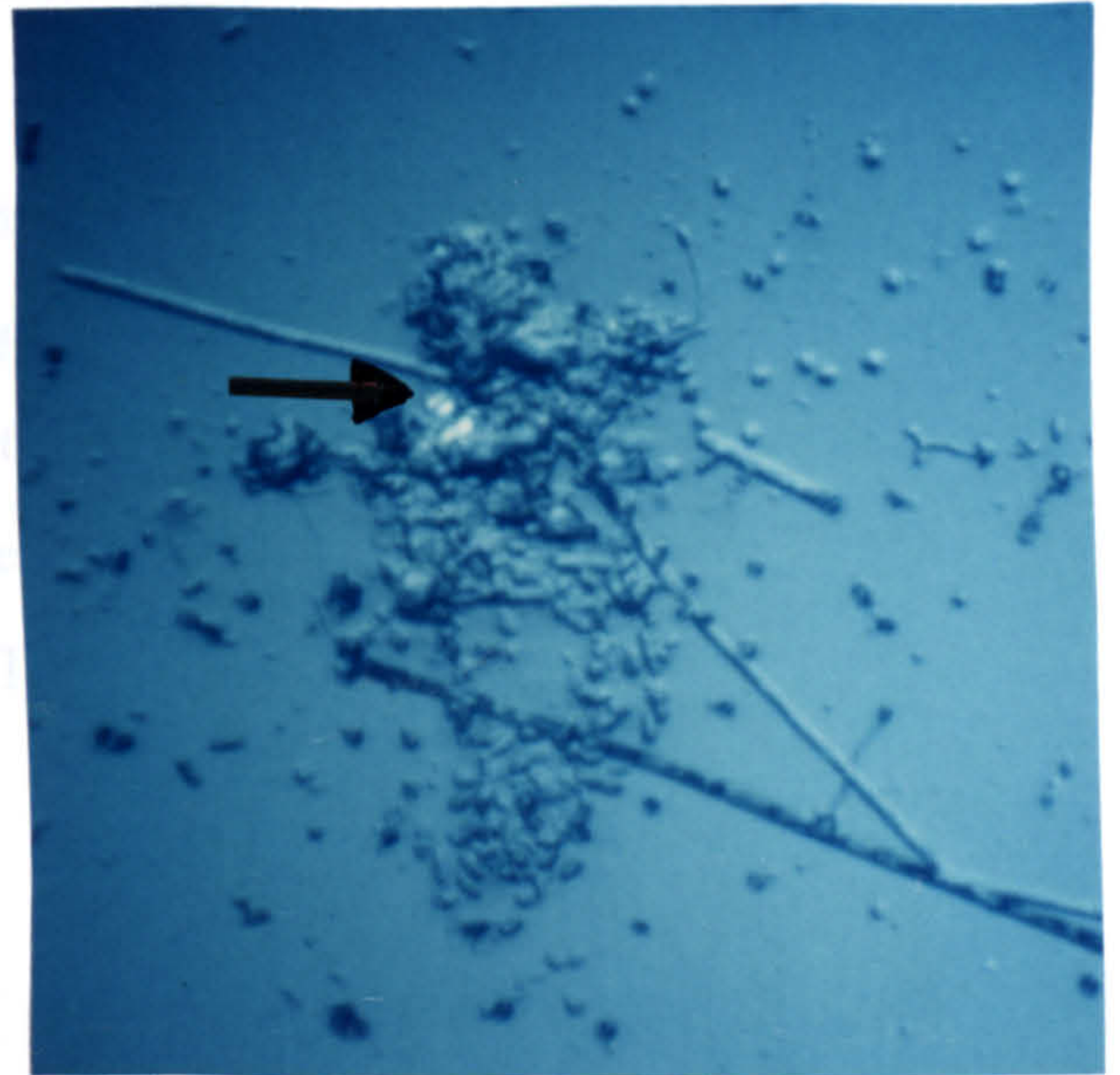
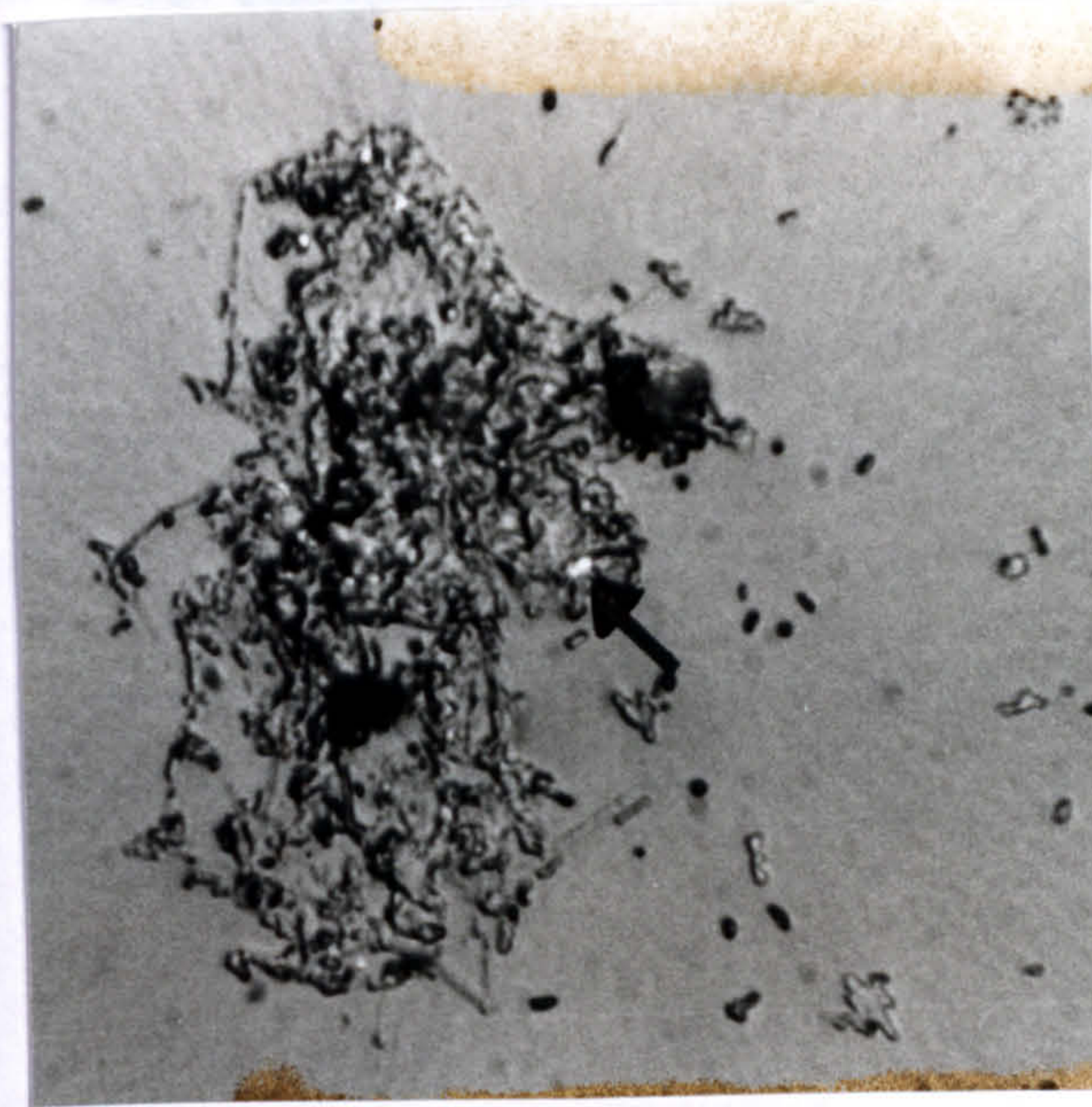
The use of gold labelling to detect legionellae in the environmental model samples with DIC microscopy allowed the simultaneous observation of total biofilm flora and the labelled legionellae, so that estimation of the proportion of legionellae in the flora population was practical. The gold-labelled legionellae were quickly and easily recognisable in the biofilm generated in the tap water. Some calcium carbonate was deposited onto the surface of the biofilm at 40 °C and had a goldlike diffuse appearance under episcopic DIC light. However, these deposits were clearly morphologically different from those of the bacterial cells, and the cells could be easily distinguished. Culture of biofilm flora on BCYE or low-nutrient R2A showed that the legionellae composed a low proportion (less than 2 %) of the total biofilm flora; however, the immunogold labelled *L. pneumophila* cells were readily detectable within the biofilm in comparable proportions.

The immunogold-labelled biofilms that were on glass surfaces were also examined under transmitted incident light. The immunogold-labelled cells had a dense black appearance, which made them more difficult to differentiate from the biofilm flora with this method than with the DIC microscopy.

FITC-labelling

The legionellae in the biofilm were successfully labelled apple green by the alternative FITC method, and there was minimal non specific binding. The biofilm contained some organisms which autofluoresced red, probably due to chlorophyll-containing cyanobacteria or algae; calcium carbonate deposits were diffusely and nonspecifically labelled a light apple green. As with the gold-labelled legionella-containing biofilm, non-legionella fluorescent microstructures within the biofilm were easily recognised by their difference in colour and morphology so could be discounted. The positive control pure culture of legionella was labelled strongly, and the bacterial cells appeared apple green on a black background. The negative controls did not stain.

Figure 3.8 Micrographs of immunolabelled *L. pneumophila* in 7 day old biofilms formed at 40 °C, showing single cells and microcolonies of the pathogen.



3.3.7 Virulence of *L. pneumophila* in the chemostat model

Of the four guinea pigs exposed to an inoculum containing 1×10^{10} , two died after two days and the remaining two died the next day. The numbers retained in the lung was 6.25×10^6 . Three of the animals infected with the inoculum containing 1.0×10^9 died after three days. Of the animals receiving the inoculum containing 1.0×10^8 one died after 4 days and one more died after 5 days. The assumed number retained in the lungs resulted in an L.D.₅₀ of 4.3 (log data).

3.4 DISCUSSION

The appearance of microcolonies in the biofilm was suggested by the clustering of bacteria of similar morphology and the appearance of bands of different coloured microbes in the biofilm. The normal growth habit of bacteria is by colony formation and this morphology reflects this mode of growth. The zoning of coloured bands of bacteria would indicate that symbiosis was possible in the biofilms.

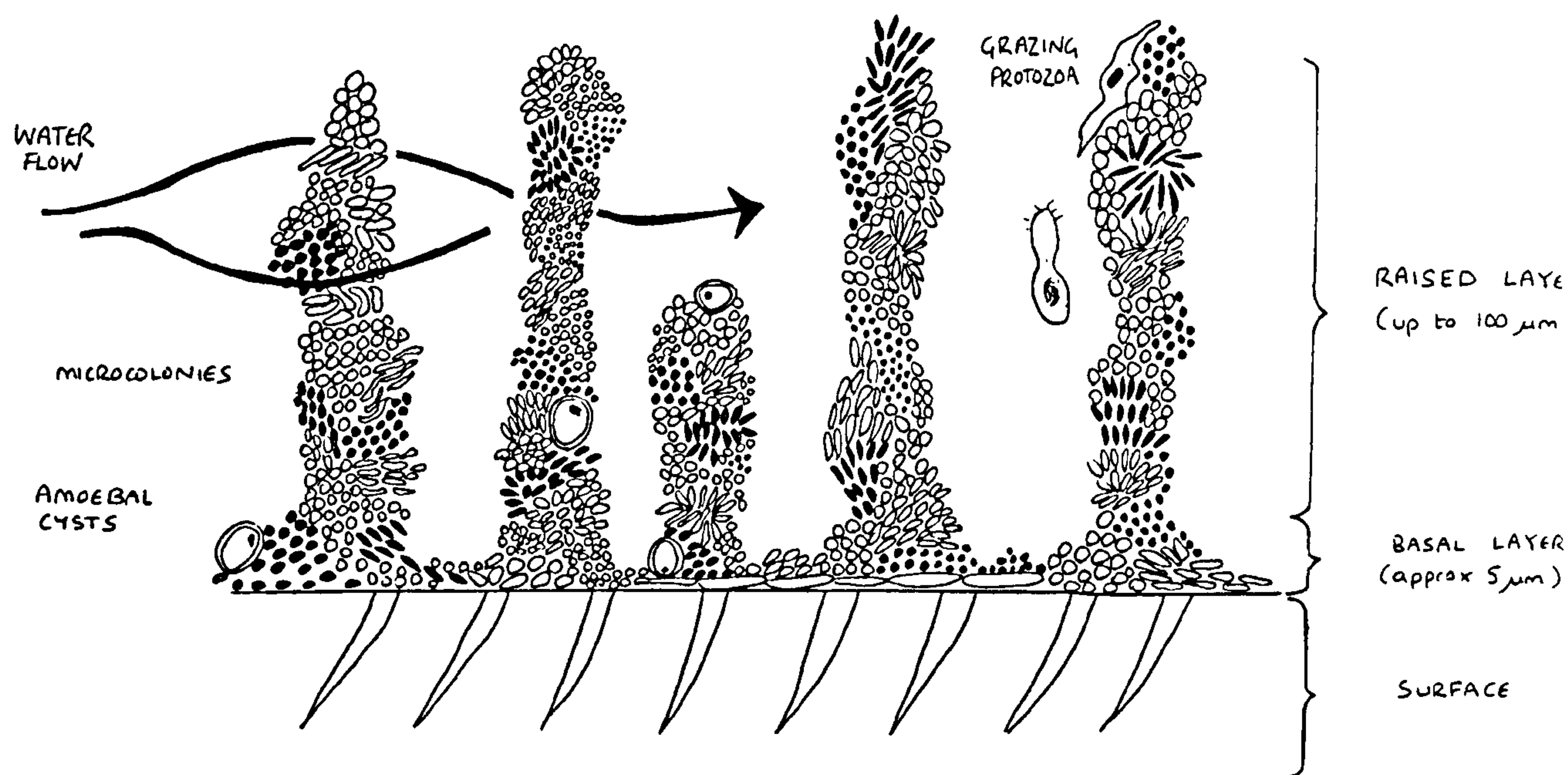
Immunogold labelling of *L. pneumophila* in the bacterial consortium of the biofilm allowed simultaneous observation of the *L. pneumophila* and the unlabelled biofilm cells. There is now considerable evidence that legionellae grow intracellularly within free living amoebae (Wadowsky *et al.*, 1991), and it is now accepted that amoebae and other protozoa provide an important means of increasing the numbers of *L. pneumophila* within the aquatic environment. The presence of small cells of *L. pneumophila* in tight microcolonies among the biofilm flora suggest that the organisms are actively growing within the consortium and that the growth of the legionellae could be sustained in the absence of host organisms. Although the biofilm generated at lower temperatures contained a diverse range of protozoa, at 40 °C protozoa were not detectable by either culture on bacterial lawns or light or electron microscopy. At the higher temperatures, therefore, the legionellae may continue to grow extracellularly as part of the complex microbial consortia, receiving essential nutrients from several genera of bacteria. In this regard *L. pneumophila* has been described under some laboratory conditions to be a microaerophile (Mauchline and Keevil, 1991). Extracellular growth within microcolonies of aerobic respiring aquatic species may provide a suitable low-oxygen environment for enhanced growth.

It is likely that both the biofilm microflora and the protozoan hosts play important roles in the extracellular and intracellular amplification of numbers of *L. pneumophila* within

the aquatic environment. A proposed structure of a biofilm containing *L. pneumophila* is presented in Figure 3.9. Further work with differing temperature and incubation times may provide more information of the interactions that exist between the biofilm flora, grazing protozoa and *L. pneumophila*.

The immunological staining methods described here could be useful for the routine detection and visualisation if coupons of appropriate plumbing materials were inserted into cooling towers or biofilm sampling devices were fitted into pipe systems. A modification of the method may also be useful for the detection of other aquatic microorganisms, including *Pseudomonas aeruginosa*, *Aeromonas hydrophila* and *Escherichia coli* if appropriate monoclonal or polyclonal antibodies are available.

Figure 3.9 Three dimensional structure of a biofilm containing *L. pneumophila*, where growth could be achieved intracellularly be amoebae and extracellularly by nutrient supply be the bacterial consortium.



CHAPTER 4

EFFECT OF MATERIALS ON BIOFILM AND PLANKTONIC GROWTH

4.1 INTRODUCTION

In the United Kingdom the influence of materials on water quality is controlled by the application of the British Standard BS 6920. The standard ensures that the material does not contribute to poor water quality by producing unacceptable taste or odours, by releasing toxic chemicals or by encouraging microbial growth. The test for the ability to support microorganisms uses a naturally occurring, river-derived, inoculum and a water of known chemistry to determine bacterial growth indirectly using oxygen depletion. The procedure was developed to produce a low cost test which could readily be applied to a large number of materials.

The contribution to improvement of water quality that BS 6920 has achieved is without question, due to the removal of those materials that would leach nutrient into the water system. However, some materials support microbial growth without contributing nutrients and therefore there are several other factors which could be considered when evaluating materials for use in potable water systems. These include the rate of biofilm development on different materials and which organisms are present in the biofilms on various surfaces. Of particular interest is the proportion of pathogens which occur within developing biofilms. Although natural latex is known to encourage bacterial growth it is not known to what extent the material encourages growth of particular bacteria and what proportion of the resulting biofilm flora is potentially pathogenic, for example *L. pneumophila*. In addition, materials that do not provide nutrients for bacterial growth and comply with BS6920, will allow biofilms to form on their surfaces without contributing nutrients. These materials may inadvertently be sustaining a biofilm containing high numbers of *L. pneumophila*.

Plastic surfaces are known to leach metal ions at a sufficiently low level to prevent a

toxic effect to bacteria , but they could possibly contribute ions essential for enzyme function. Bacterial cells directly in contact with the materials are more likely to take up the ions. The plasticisers and other components of the pipe material may also be directly utilisable by some of the population of microorganisms in the biofilm and so contribute to the consortium as a whole. The biofilm flora would be modified if alternative nutrients were added to the community and resulting populations may vary in their ability to support *L. pneumophila*.

Although there has been little study of the influence of material on the biofilm formation and inclusion of *L. pneumophila* the work already completed suggests that there is some disagreement. Some of the previous work would indicate that the colonisation of different plumbing materials would not be significantly different (Pederson, 1990). The colonisation of surfaces by *L. pneumophila* has been shown to be unrelated to the substrate by Wright *et al.* (1989). However, the conditions which the materials were colonised were unrepresentative of the conditions found in potable water systems. The study used pure cultures of *L. pneumophila* rather than mixed consortia, added 10 % algal extract (the legionellae were incapable of growing in monoculture in sterile unsupplemented water) and the system was recirculated to achieve artificially high levels of colonisation.

Other work would suggest that materials did influence colonisation, McEldowney & Fletcher (1986) for example, found that the hydrophobic/hydrophilic nature of the surfaces affected the attachment of aquatic bacterial species to the surfaces.

Schofield and Locci (1985) examined colonisation of different materials by *L. pneumophila* in a short term study. The data suggested that the surfaces of copper were poorly colonised by cultures of *L. pneumophila* in a recirculating model system using tap water as the nutrient source. Rubber components were heavily colonised forming dense biofilm with high numbers of *L. pneumophila*. The model system recirculated the water for four months prior to observing the *L. pneumophila* by fluorescein-labelled

monoclonal antibodies. Further evidence for the impact of material selection was provided by West *et al.* (1990) which showed that copper was inhibitory to the colonisation and growth of *L. pneumophila*.

The objective of this experimental work was to determine what influence materials used for the construction of water systems may have on water quality. The biofilm model system developed in Chapter 3 was used to study the possible influences of material on the colonisation of biofilms and the inclusion of legionella into these biofilms. The use of the model system for this study ensured that the conditions of the test were identical for all of the materials and that the only factor which was changed was the material. The chemostat design provided a constant flow of microorganisms to the biofilm generating vessel, thus ensuring any modification of the microbial population by previous environmental conditions during experiments could be negated.

The progressive incorporation of legionellae into the biofilms on a range of commonly used plumbing materials was monitored so that a comparison could be made of total biofouling and inclusion of the pathogen. The study was focussed on the impact of material on biofilm population development to determine if colonisation and growth of *L. pneumophila* was influenced by material selection.

4.2 MATERIALS AND METHODS

4.2.1 Colonisation of plumbing materials

A diverse range of plumbing materials was compared for the ability to support biofilm and a population of *L. pneumophila* at 30°C using the Thames river water as the nutrient for the chemostat model. The materials tested included elastomeric materials used for sealing water systems, sections of plastic tube and sections of steel tube.

Latex (natural latex) is a natural plant product that was extensively used to make sealing materials (eg tap washers) in pipes and taps carrying potable water. However, this material supports the growth of microorganisms which will cause a deterioration in water quality and biodeterioration of the seals. Consequently natural latex was withdrawn from use. The material provides a good positive control representing a material unsuitable for use in the water supply system. A wide range of synthetic elastomeric materials are now available for seal manufacture including ethylene-propylene copolymers. A newly developed ethylene-propylene material was supplied for testing and this was included in the trial.

Plastic surfaces are widely used in domestic water systems and plastic tube that is commercially available was tested in the model system. Plastics tested included polyethylene, unplasticised PVC (PVCu), chlorinated PVC (PVCc), and polypropylene. Pipe made from PVCc is used for hot and cold water up to a maximum continuous temperature of 85 °C. The sample was manufactured by Hunter Genova. The PVCu and polyethylene tube is commonly used for cold water applications, PVCu pipe sample was supplied in sections by Wavin Ireland Ltd, and the medium density polyethylene tube samples was extruded by Uponer. Polypropylene is used to provide robust, chemically tolerant tube and the sample tested was produced by Glynwed Tubes and

Fittings Ltd. under the trade name of Vulcathene. Mild steel and stainless steel 361 tube are commonly used in contact with water within cooling towers and other industrial plant.

Each material (with the exception of stainless and mild steel) was tested independently. Samples of the plumbing material, along with the glass control tiles, were inserted into biofilm generation vessel of the chemostat model as described in 2.2 to allow biofilm to develop over a 28 day period. The chemostat was operated at 30°C with oxygen being maintained at 20% DOT. In each of the material tests the planktonic and biofilm development were monitored in order to determine the growth of the total flora and the legionellae in that population (Section 2.3). The biofilms on the materials were also stained with osmium tetroxide and gold coated following removal from the chemostat so that they could be viewed under the scanning electron microscope (as described in Sections 2.6 and 2.7).

4.2.2 Leaching of nutrient from materials

Results from the colonisation of the different materials showed plastic and elastomeric materials were more rapidly colonised than glass surfaces. This observation suggested the possibility that nutrients were available at the material surfaces and that the bacteria were able to grow more abundantly, particularly at the onset of the experiment when maximum nutrient would be available. The glass control surfaces were slightly more colonised in the presence of the materials than when put into the chemostat alone. In order to determine whether nutrient was leaching into the planktonic phase the total organic carbon of the water was determined using a total organic carbon analyser (TOC). Sections (0.3cm^2 surface area) of the sterile plastic material were inserted into 10 ml of sterile distilled water and shaken for 3 days to allow nutrients to be released

into the water. The controls were glass tiles and water alone. After 3 days the TOC was determined to see if leaching had occurred using the methods described in 2.8.

4.2.3 Effect of heating on recovery of *L. pneumophila*

The standard method for detection of *L. pneumophila* in water samples includes acid or heat pretreatment in order to reduce background flora to enable the less rapidly growing legionellae to be recovered. Due to the small volume of biofilm sample available acid treatment was not used. Legionellae were enumerated by inoculating the sample directly on to the media directly and after heating. Duplicate samples of the planktonic culture were heated at 50°C for 5, 10, 15, 20, 25, and 30 min intervals and legionellae enumerated on BCYE.

4.3 RESULTS

4.3.1 Planktonic microorganisms

The culture in the test vessel of the model system contained a total microbial flora of 10^5 - 10^6 cfu ml⁻¹, with numbers of *L. pneumophila* being 10^3 - 10^4 cfu ml⁻¹ (Table 4.1) during the experiments. The diversity of microorganisms in the planktonic phase was maintained over the experiments with all of the species that were initially present being maintained in culture.

On inclusion of latex into the vessel the population of planktonic microorganisms increased from 5.0×10^5 to 2.95×10^6 cfu ml⁻¹, with concomitant reductions in the numbers of *L. pneumophila* (3.0×10^3 cfu ml⁻¹). Numbers of the pathogen then increased to 3.0×10^4 cfu ml⁻¹, accounting for 0.2-0.9% of the total microbial flora. The non-legionella population stabilised around 3.5×10^5 cfu ml⁻¹. Similar effects were observed following the insertion of ethylene-propylene into the aqueous phase. The total population increased to 7.7×10^5 cfu ml⁻¹ and *L. pneumophila* numbers were reduced to 1.8×10^3 cfu ml⁻¹ (representing >0.9% of the population over the experiment).

The introduction of polypropylene into the model system resulted in an initial increase in the aquatic population from 5.1 to 9.5×10^5 cfu ml⁻¹. *Legionella pneumophila* numbers also increased to 9.5×10^3 cfu ml⁻¹ and these were >2.9% of the total population. When coupons of PVCc were added to the culture, corresponding increases in the total planktonic population (5.0×10^4 cfu ml⁻¹) and *L. pneumophila* numbers (3.0×10^3 cfu ml⁻¹) occurred, with the pathogen representing up to 18% of the flora. In the presence of PVCc the number of aquatic microorganisms increased over the duration of the experiment to 2.75×10^6 cfu ml⁻¹ at day 28. *Legionella pneumophila* increased in a similar manner to a maximum of 3.95×10^3 cfu ml⁻¹ (1.5% of the

population). Steel coupons caused an increase in the total planktonic population to 8.6×10^5 cfu ml⁻¹ and *L. pneumophila* numbers increased to 9.5×10^3 cfu ml⁻¹. Although polyethylene caused similar increases in the total flora when introduced into the planktonic phase (5.0×10^5 cfu ml⁻¹), the *L. pneumophila* numbers were reduced during colonisation. The minimum numbers of *L. pneumophila* were 4.05×10^3 cfu ml⁻¹ and represented 1.5% of the total population.

Table 4:1 Comparison of the numbers of microorganisms occurring in the biofilm and planktonic phases of the model system.

Material	mean in biofilm (numbers cm ⁻²)	mean in planktonic (numbers ml ⁻¹)	ratio of biofilm to planktonic
NON-LEGIONELLAE			
Stainless steel	2.13x10 ⁵	2.23x10 ⁵	0.96
Polypropylene	4.45x10 ⁵	4.48x10 ⁵	1.01
PVCc	5.14x10 ⁵	6.20x10 ⁵	0.83
PVCu	6.23x10 ⁵	3.22x10 ⁴	10.35
Mild steel	1.69x10 ⁶	2.23x10 ⁵	7.58
Polyethylene	2.75x10 ⁶	1.62x10 ⁶	1.70
Ethylene-propylene	1.08x10 ⁷	7.45x10 ⁵	14.47
Latex	5.50x10 ⁷	1.87x10 ⁶	29.4
LEGIONELLAE			
Stainless steel	1.03x10 ⁴	5.30x10 ³	1.94
Polypropylene	2.10x10 ⁴	3.42x10 ³	6.14
PVCc	2.24x10 ⁴	1.23x10 ³	18.21
PVCu	7.75x10 ³	1.06x10 ³	7.31
Mild steel	2.06x10 ⁴	5.30x10 ³	3.89
Polyethylene	6.76x10 ³	6.68x10 ³	1.01
Ethylene-propylene	1.44x10 ⁵	1.80x10 ³	80
Latex	2.20x10 ⁵	1.38x10 ⁴	12.2

Means were calculated from all values determined over 1, 4, 7, 14, 21 and 28 days.

4.3.2 Biofouling of plumbing materials

All of the materials were rapidly colonised by microorganisms following insertion into the aquatic model system, with a minimum of 5.24×10^4 cfu cm⁻² on the stainless steel surface after only 24h (Figure 4.8). Latex and ethylene-propylene surfaces were the most rapidly biofouled materials supporting populations of 8.9 and 1.4×10^7 cfu cm⁻² after an equivalent time period (Figures 4.1 and 4.2). Stainless steel and the plastic materials supported biofilms which contained 10^5 - 10^6 cfu cm⁻² after 24h (Figure 4.7).

Latex and ethylene-propylene remained the most heavily colonised materials for the duration of the experiment, with maximum numbers of 8.9 & 2.9×10^7 cfu cm⁻² respectively. These were found to be significantly more colonised than the other materials tested (with a confidence limit of 95%). Stainless steel supported lowest numbers of microorganisms in biofilms compared with the other materials, with a maximum of 6.45×10^5 cfu cm⁻². In contrast, mild steel (which was observed to rust) supported a biofilm which contained up to 4.95×10^6 cfu cm⁻².

Of the plastic materials, polyethylene appeared to be most heavily colonised, with 1.3×10^7 cfu cm⁻² after 4 days in the model system (Figure 4.6). The total numbers of microorganisms on the surface of the other plastics remained between 10^5 - 10^6 cfu cm⁻² for the duration of the experiment, with polypropylene (Figure 4.5) < PVCc (Figure 4.3) < PVCu (Figure 4.4).

For the duration of the experiment the elastomeric surfaces supported higher numbers of microorganisms on their surfaces than the control glass surfaces. The glass surface supported less than 1.4% of the microorganisms occurring on the latex surface and less than 7.1% of the microorganisms occurring on the ethylene-propylene surface. Glass incubated with polypropylene supported 16% of that occurring on the plastic surface

and colonisation was consistently lower for the duration of the experiment. The CPVc surfaces supported high numbers of microorganisms on their surfaces than on the glass control surfaces throughout colonisation, with glass having 35-95% of the flora present on the plastic material. Although the initial colonisation of polyethylene and PVCu was more rapid than the control glass surface this was not maintained and there was no significant difference in the colonisation of the materials and the control glass surfaces.

All of the materials surfaces (with the exception of polyethylene and PVCu) supported significantly higher total flora than their control glass surfaces (using a 95% confidence limit). The colonisation of the polyethylene and the PVCc surfaces had probabilities of equalling or exceeding z of 6% and 9% respectively, so these were outside the 5% confidence limit.

Figure 4.1 Colonisation by non-legionella of latex and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

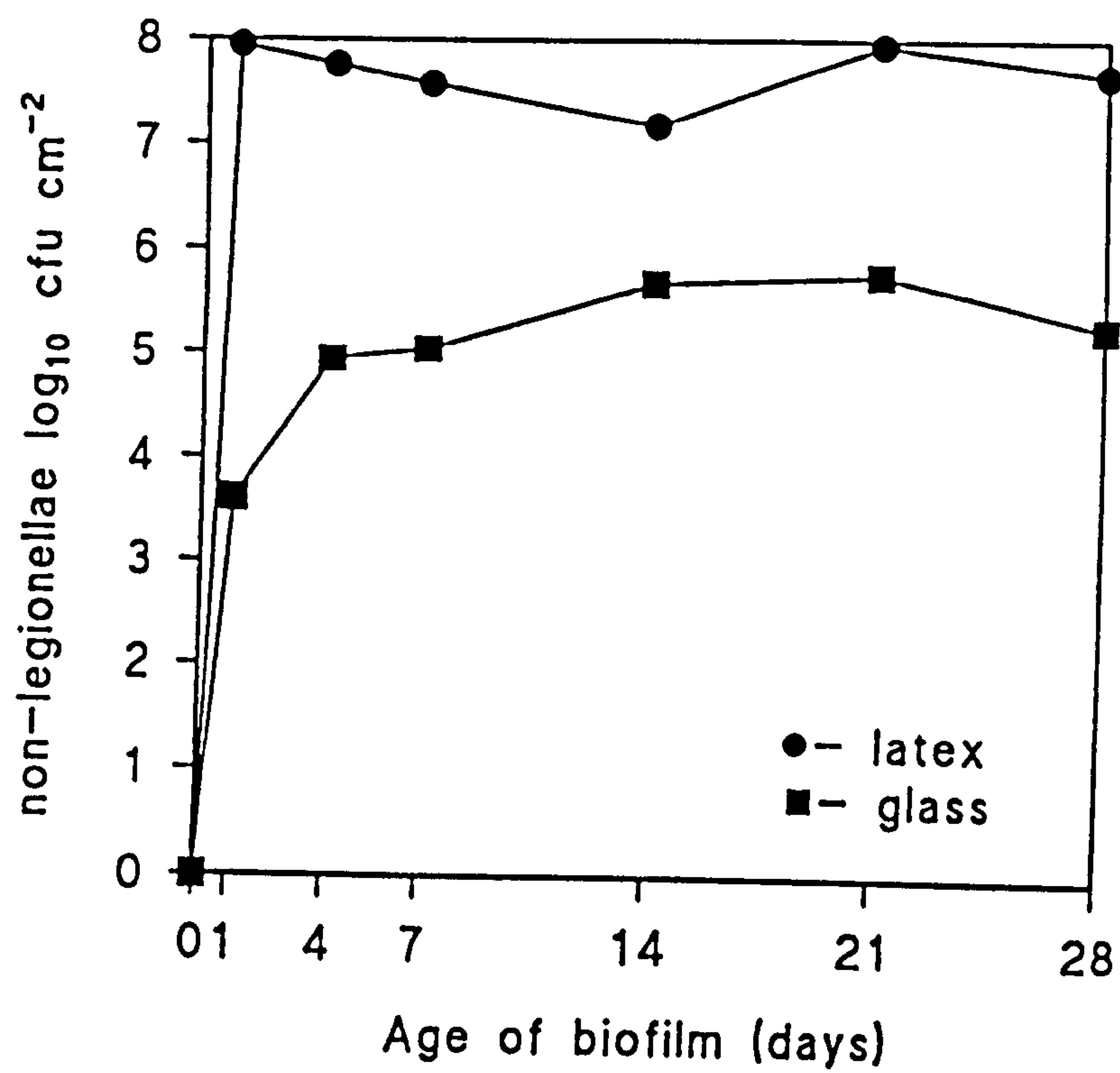


Figure 4.2 Colonisation by non-legionella of ethylene-propylene and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

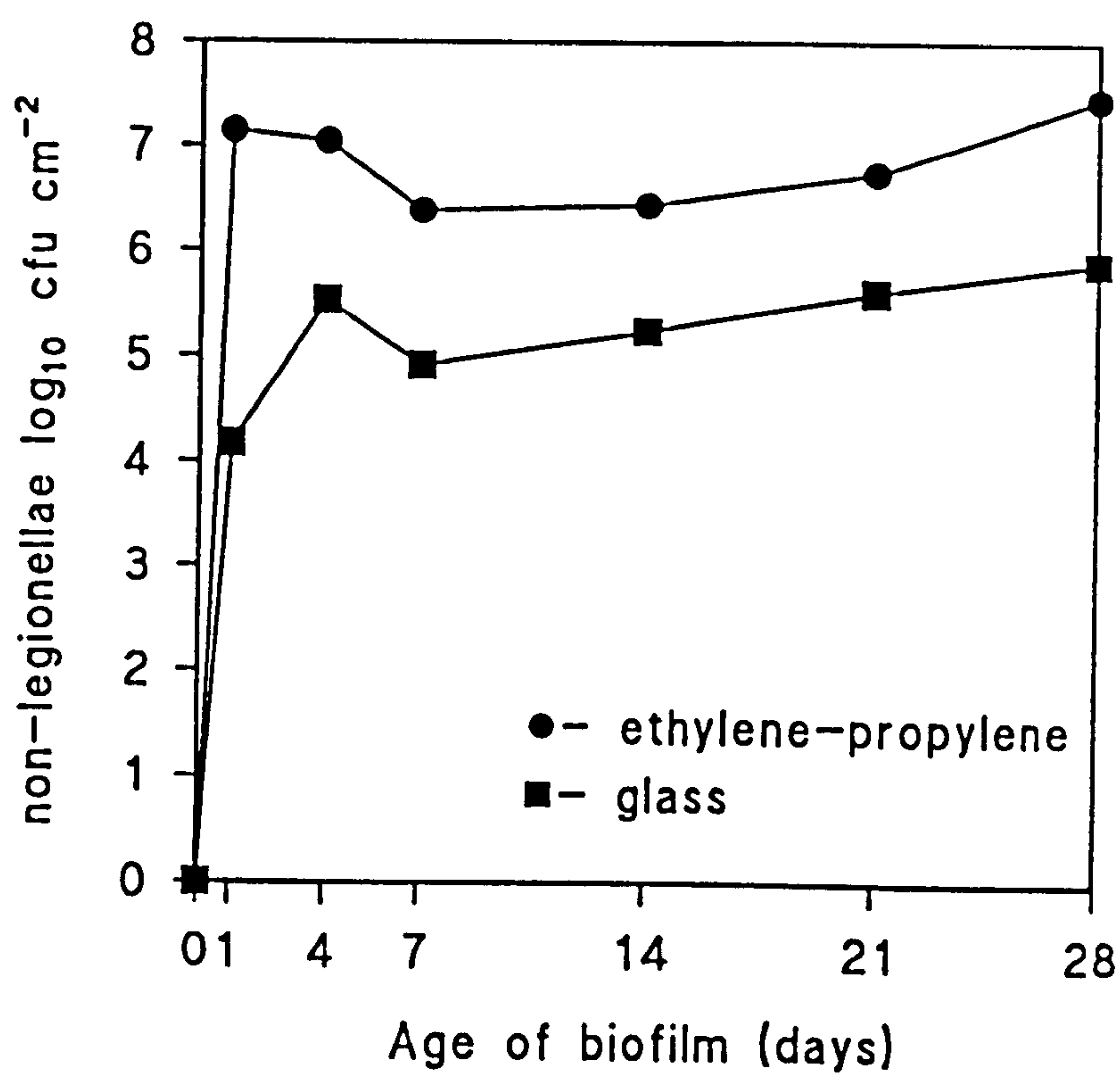


Figure 4.3 Colonisation by non-legionella of PVCc and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

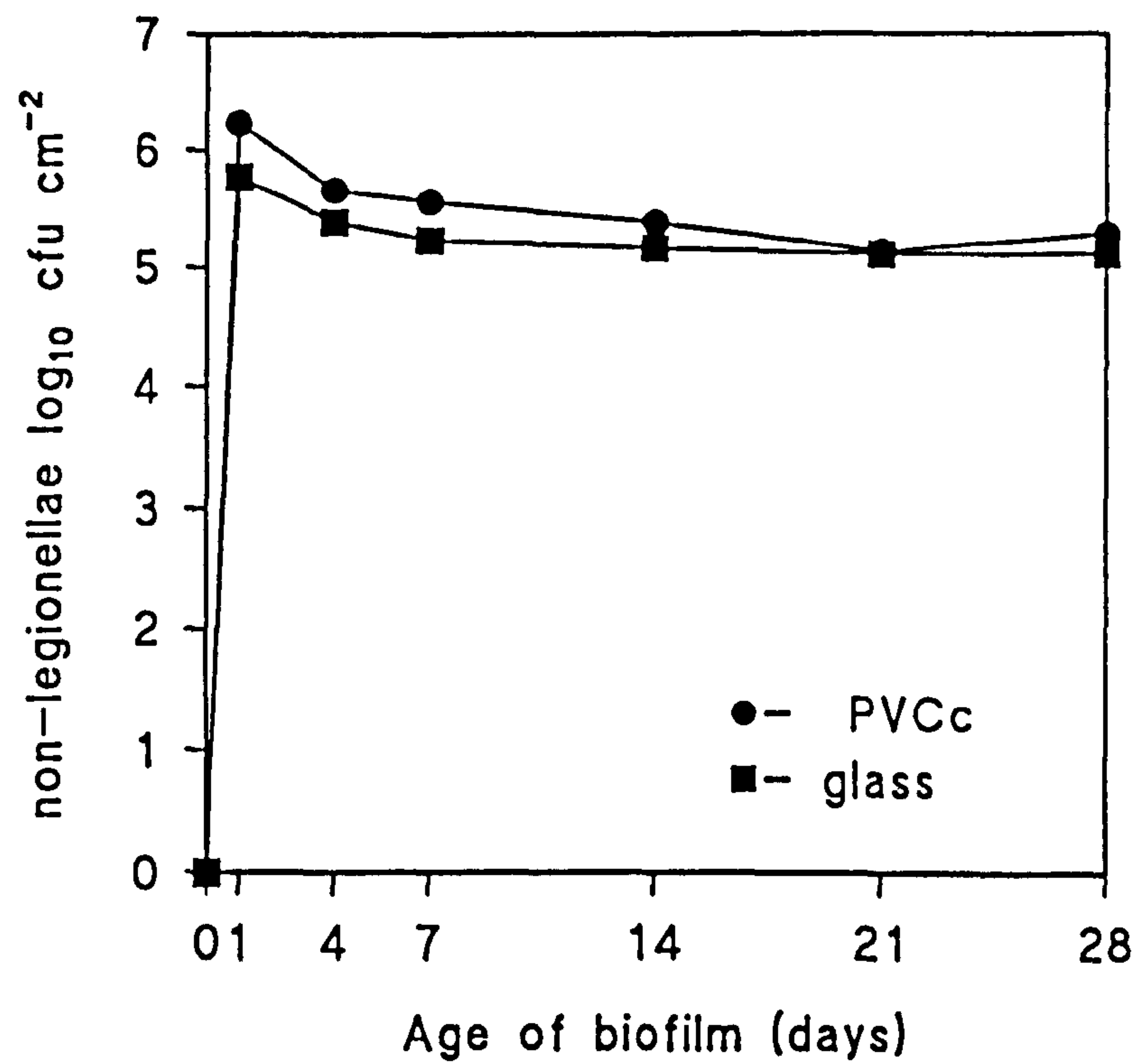


Figure 4.4 Colonisation by non-legionella of PVCu and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

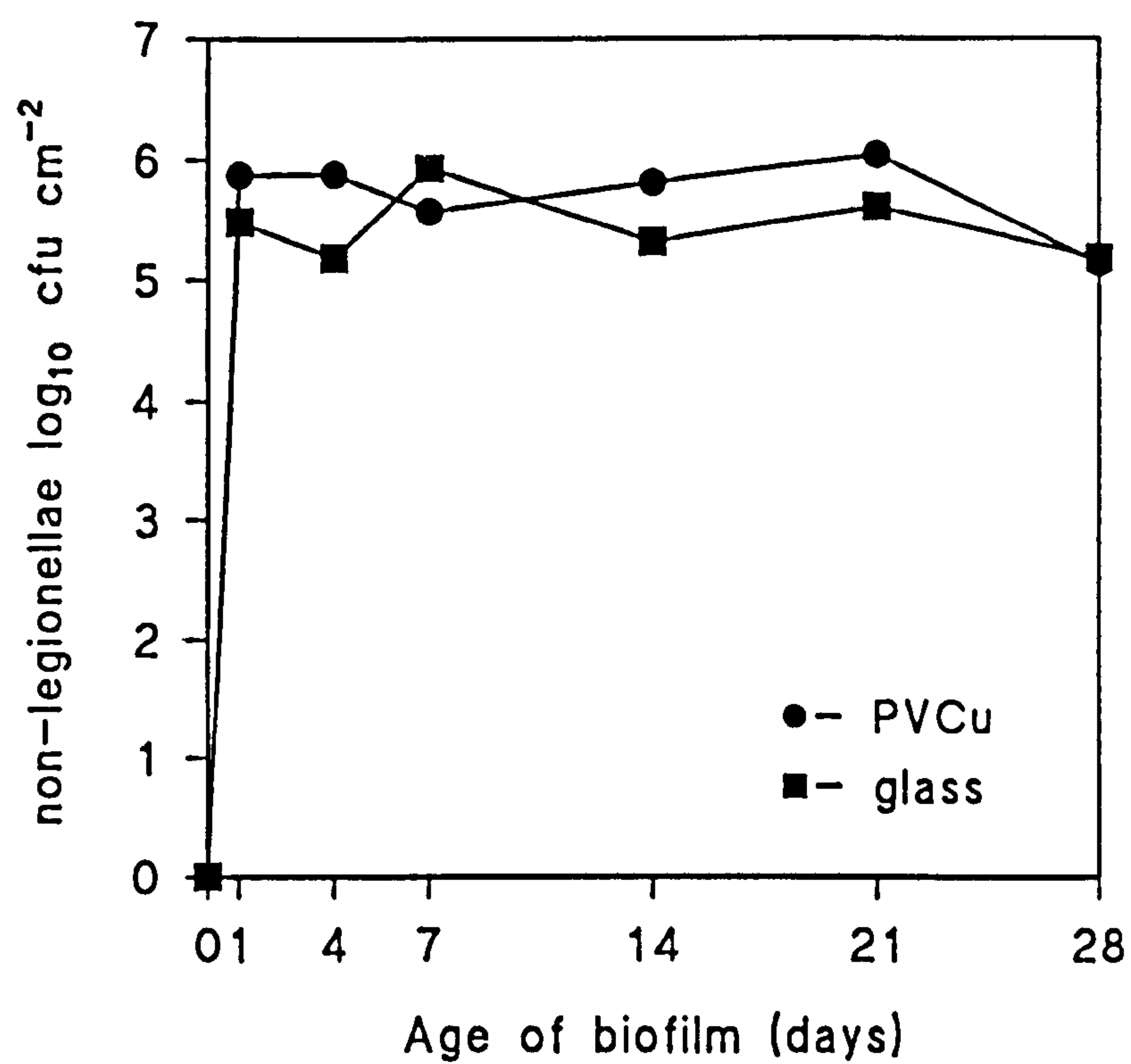


Figure 4.5 Colonisation by non-legionella of polypropylene and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

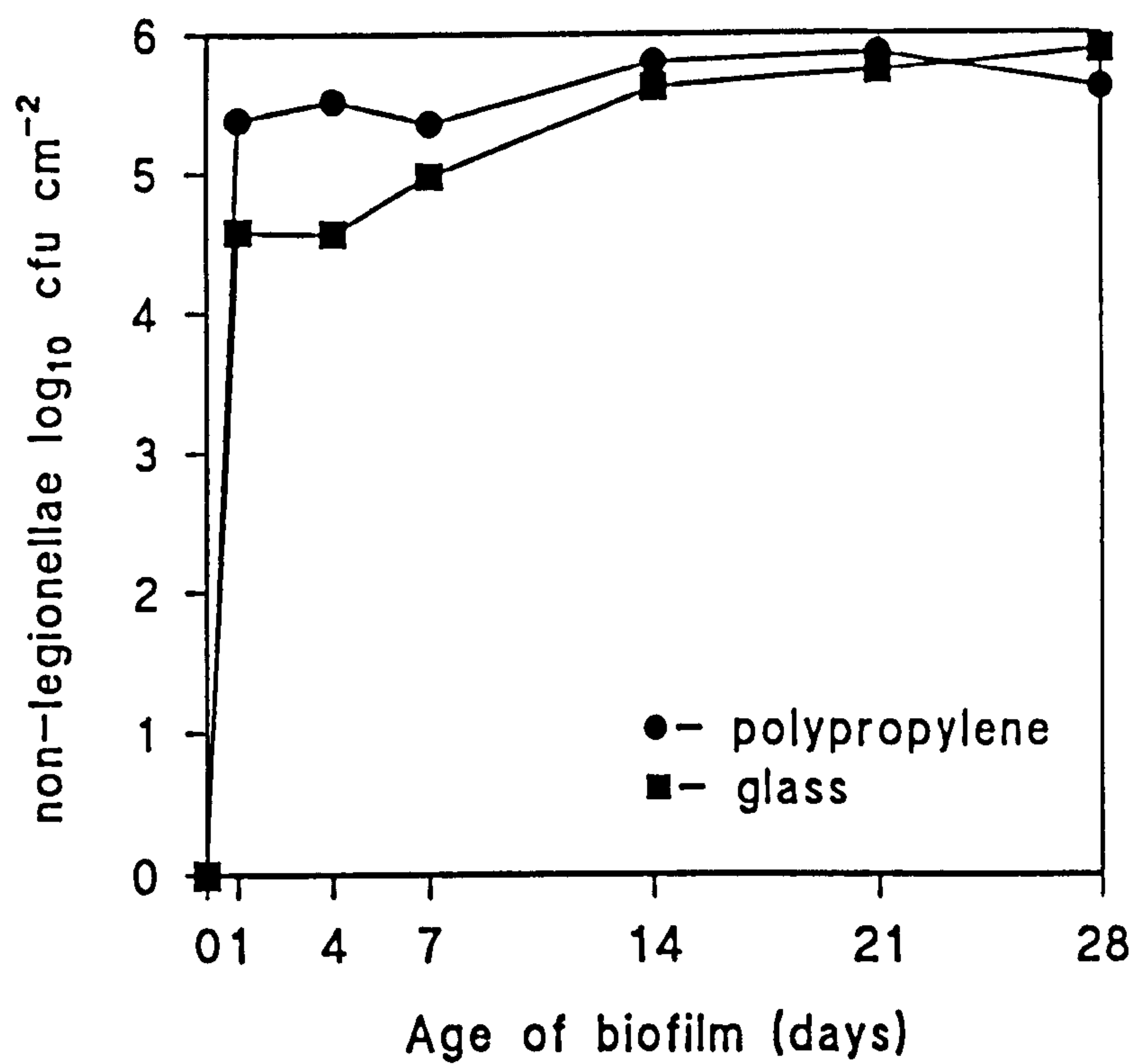


Figure 4.6 Colonisation by non-legionella of polyethylene and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

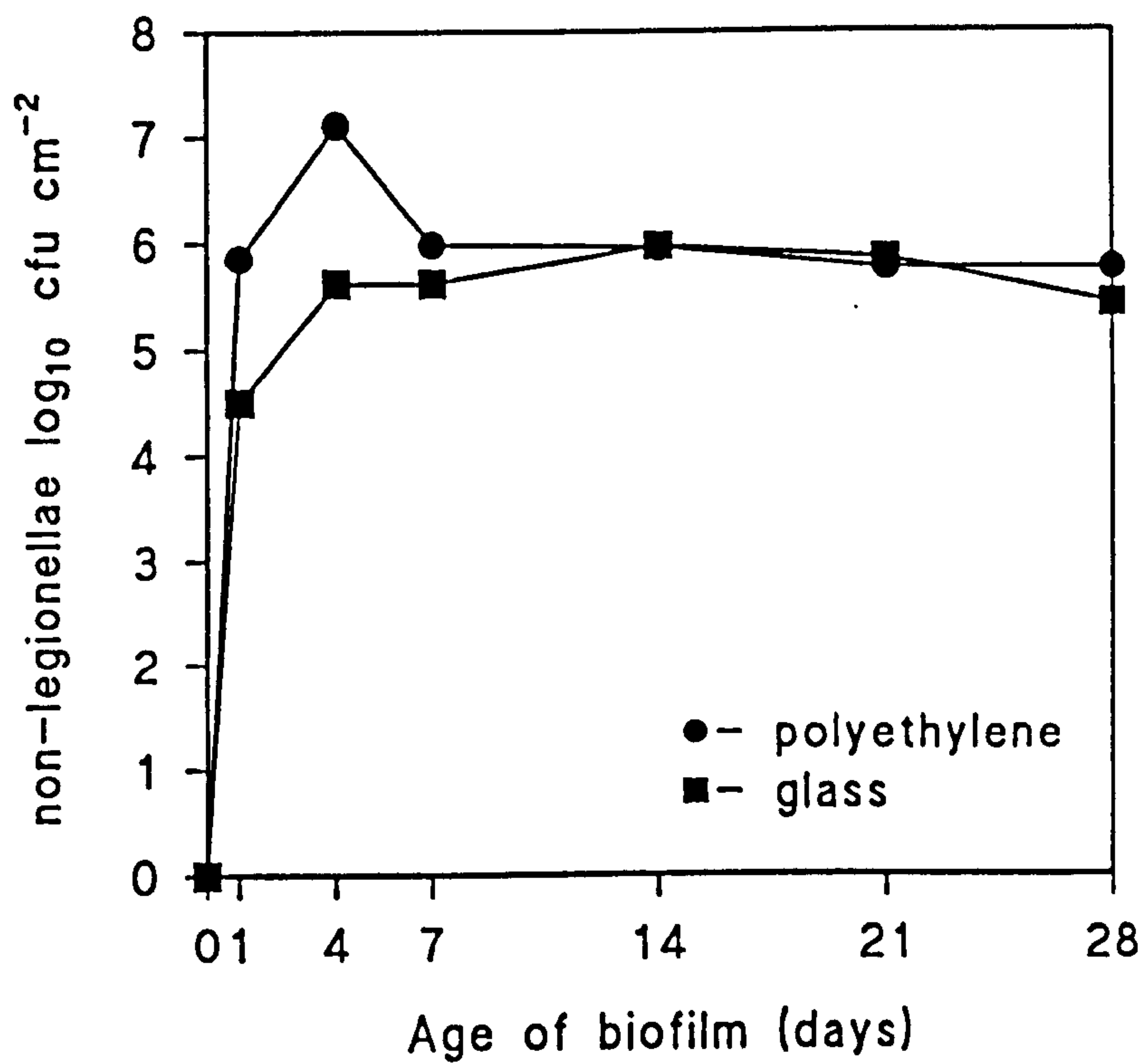


Figure 4.7 Colonisation by non-legionella of mild steel at 30°C in tap water containing a complex consortia of microorganisms. (ND denotes not done).

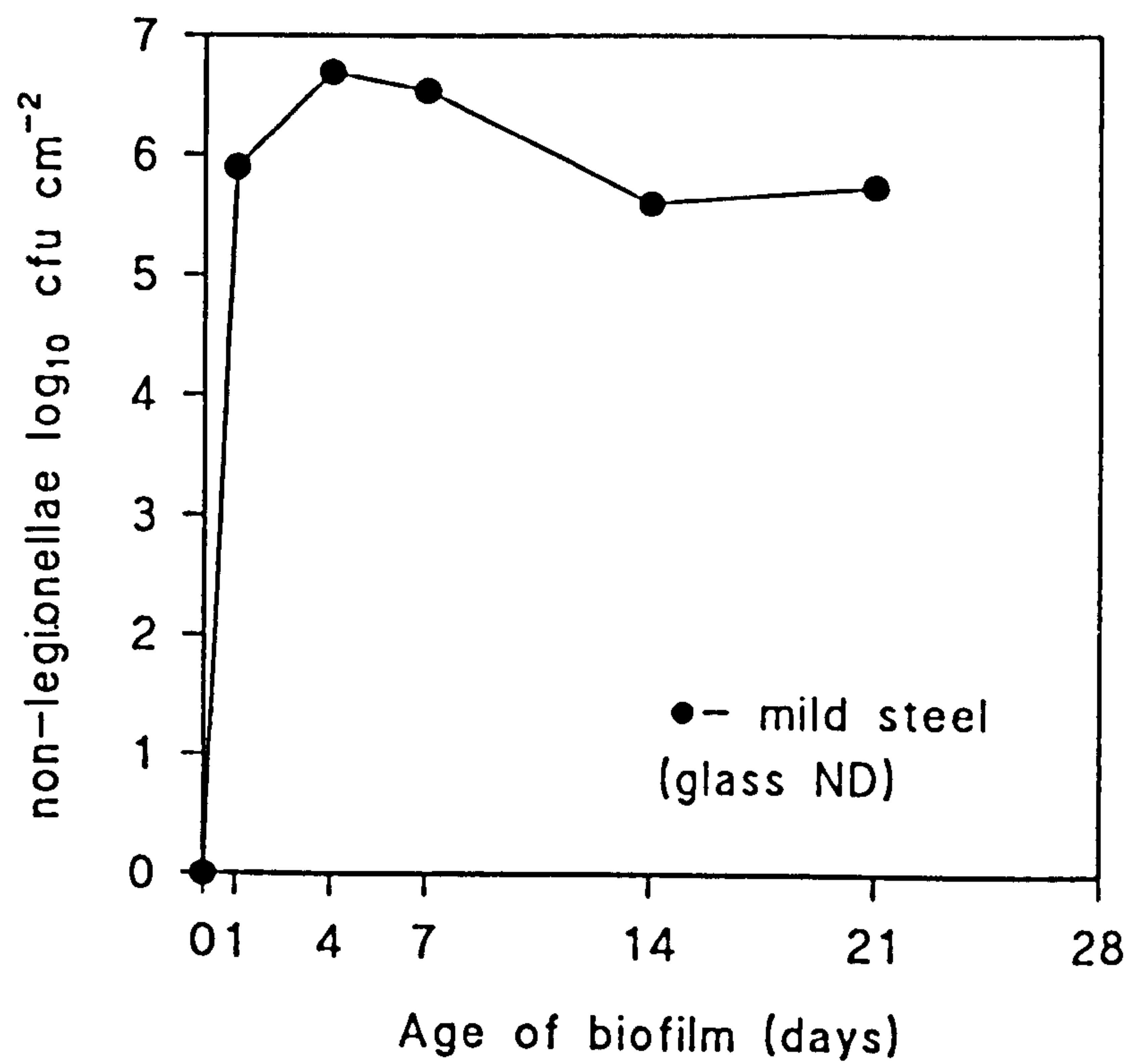


Figure 4.8 Colonisation by non-legionella of stainless steel at 30°C in tap water containing a complex consortia of microorganisms. (ND denotes not done).

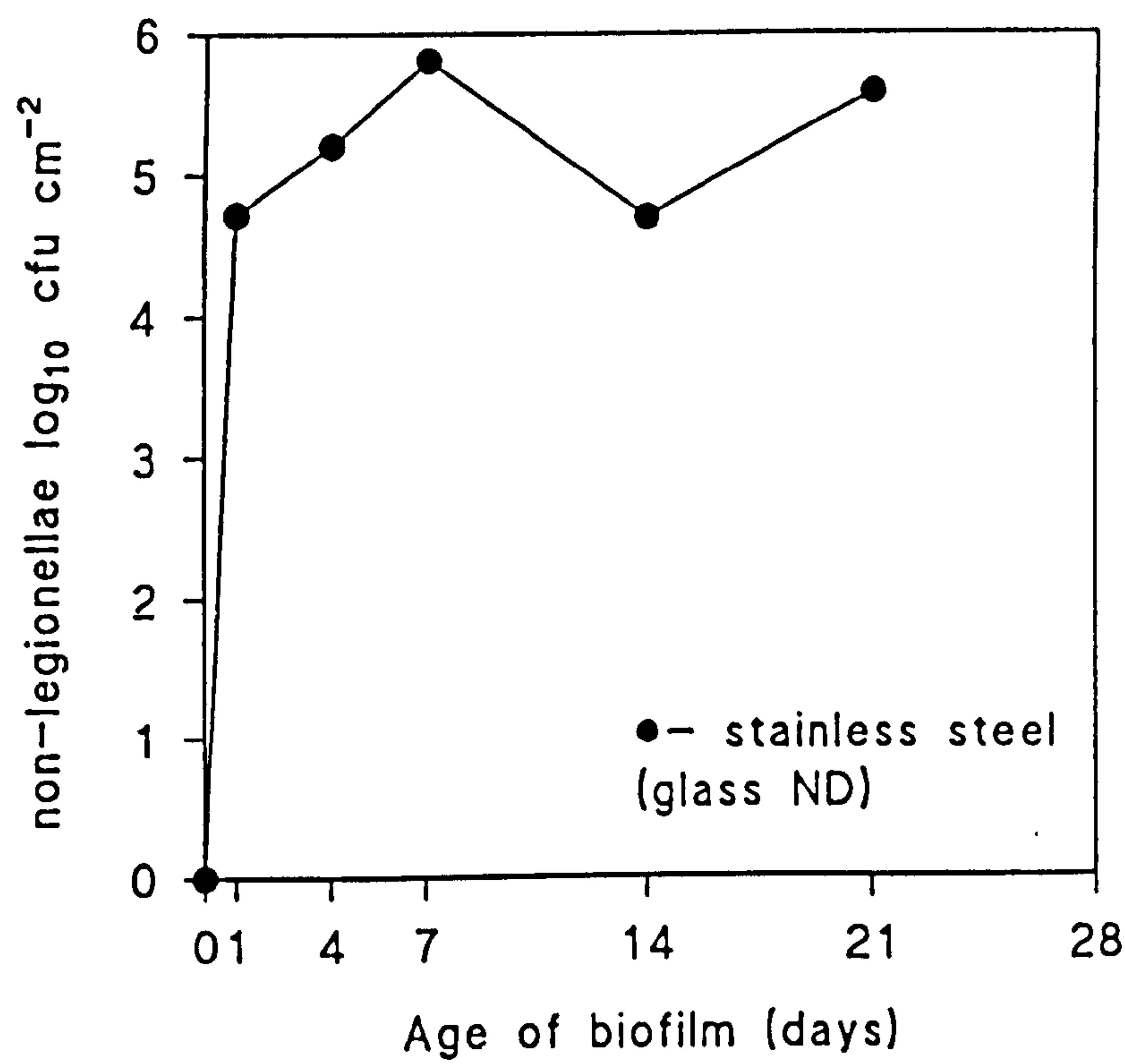


Table 4.2 Comparison of Materials for their ability to support biofilm development and colonisation by *L.pneumophila*.

Material	Mean Colonisation		Colonisation ratio	
	Total viable flora	<i>L.pneumophila</i>	total viable flora	<i>L.pneumophila</i>
Glass	1.90x10 ⁵	1.70x10 ³	1	1
Stainless steel	2.13x10 ⁵	10.3x10 ⁴	1.1	6.1
Polypropylene	4.54x10 ⁵	2.10x10 ⁴	2.4	12.4
PVCc	5.14x10 ⁵	2.24x10 ⁴	2.7	13.1
PVCu	6.23x10 ⁵	7.75x10 ³	3.3	4.6
Mild steel	1.69x10 ⁶	2.06x10 ⁴	8.9	12.1
Polyethylene	2.75x10 ⁶	6.76x10 ³	14.5	4.0
Ethylene-propylene	1.08x10 ⁷	1.44x10 ⁵	56.8	84.7
Latex	5.50x10 ⁷	2.20x10 ⁵	289	129

The colonisation ratio is the cfu of the total viable microbial flora or legionella recovered from each material when compared with that on glass.

4.3.3 Inclusion of *L. pneumophila* into biofilms

All of the materials were found to contain *L. pneumophila* in biofilms on their surfaces after only 24h exposure to the culture water containing the pathogen. The numbers of *L. pneumophila* which became included in the biofilms on the surface of the materials was unrelated to the total number of microorganisms present (Table 4.1). The biofilms on the elastomeric materials were found to contain the highest numbers of *L. pneumophila* for the duration of the experiment, with latex having 5.5×10^5 cfu cm⁻² (Figure 4.9) and ethylene-propylene having 5.0×10^5 cfu cm⁻² (Figure 4.10). However, the *L. pneumophila* accounted for a maximum of only 3.5% of the total bacterial population on the elastomeric surfaces. In contrast, several materials had a higher proportion of *L. pneumophila* within the biofilm. *L. pneumophila* accounted for up to 22% of the total microbial population of the biofilm on PVCc after 7 days in the model system (Figure 11).

The number of *L. pneumophila* varied in the biofilms which developed on the plastic surfaces. Polypropylene supported a biofilm containing up to 6.6×10^4 cfu cm⁻² of *L. pneumophila* which accounted for 10.3% of the total biofilm flora (Figure 4.13). Polyethylene contained less *L. pneumophila*, with a maximum of 2.3×10^4 cfu cm⁻² and these accounted for 4% of the total biofilm flora (Figure 4.14). The PVCu material supported a biofilm with a maximum of 1.0×10^4 cfu cm⁻² *L. pneumophila* in the biofilm which represented only 1% of the microorganisms (Figure 4.12). In contrast, the CPVc material supported a biofilm which contained 7.8×10^4 cfu cm⁻² *L. pneumophila* and accounted for as much as 15% of the total biofilm flora (Figure 4.11). The metal surfaces contained a high proportion of *L. pneumophila* within biofilms on their surfaces, with as much as 11% of the population on stainless steel and 31% of the population on mild steel being *L. pneumophila* (Figures 4.16 and 4.15).

The biofilms which formed on the plumbing materials were consistently found to contain more *L. pneumophila* than the control glass surfaces. Without exception, the materials tested had significantly higher numbers of *L. pneumophila* in their biofilms than the glass surfaces using a 95% confidence limit. The glass surfaces were found to contain less than 57% of the number of *L. pneumophila* occurring on the material surfaces.

Figure 4.9 Colonisation by *L. pneumophila* of latex and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

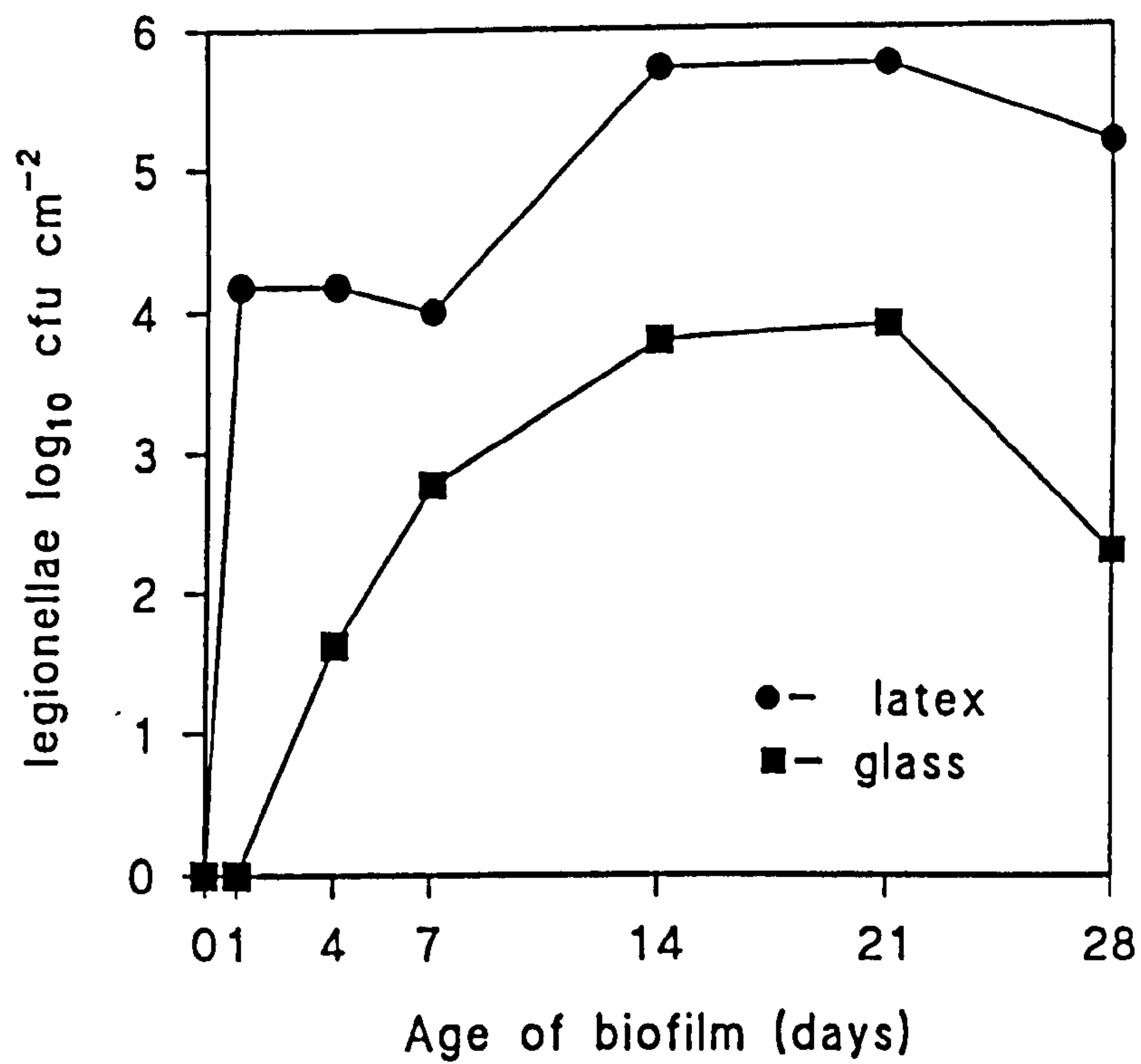


Figure 4.10 Colonisation by *L. pneumophila* of ethylene-propylene and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

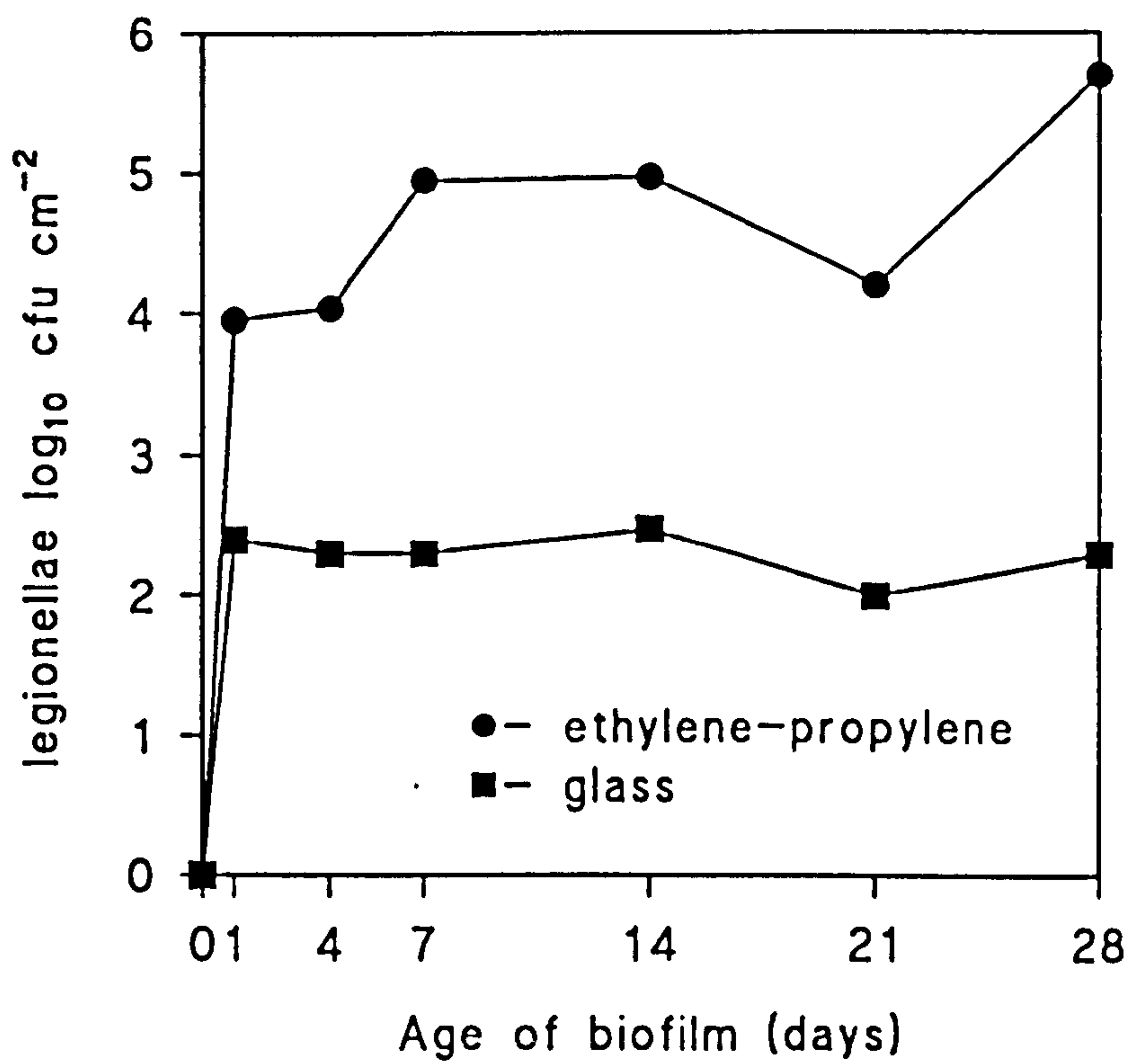


Figure 4.11 Colonisation by *L. pneumophila* of PVCc and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

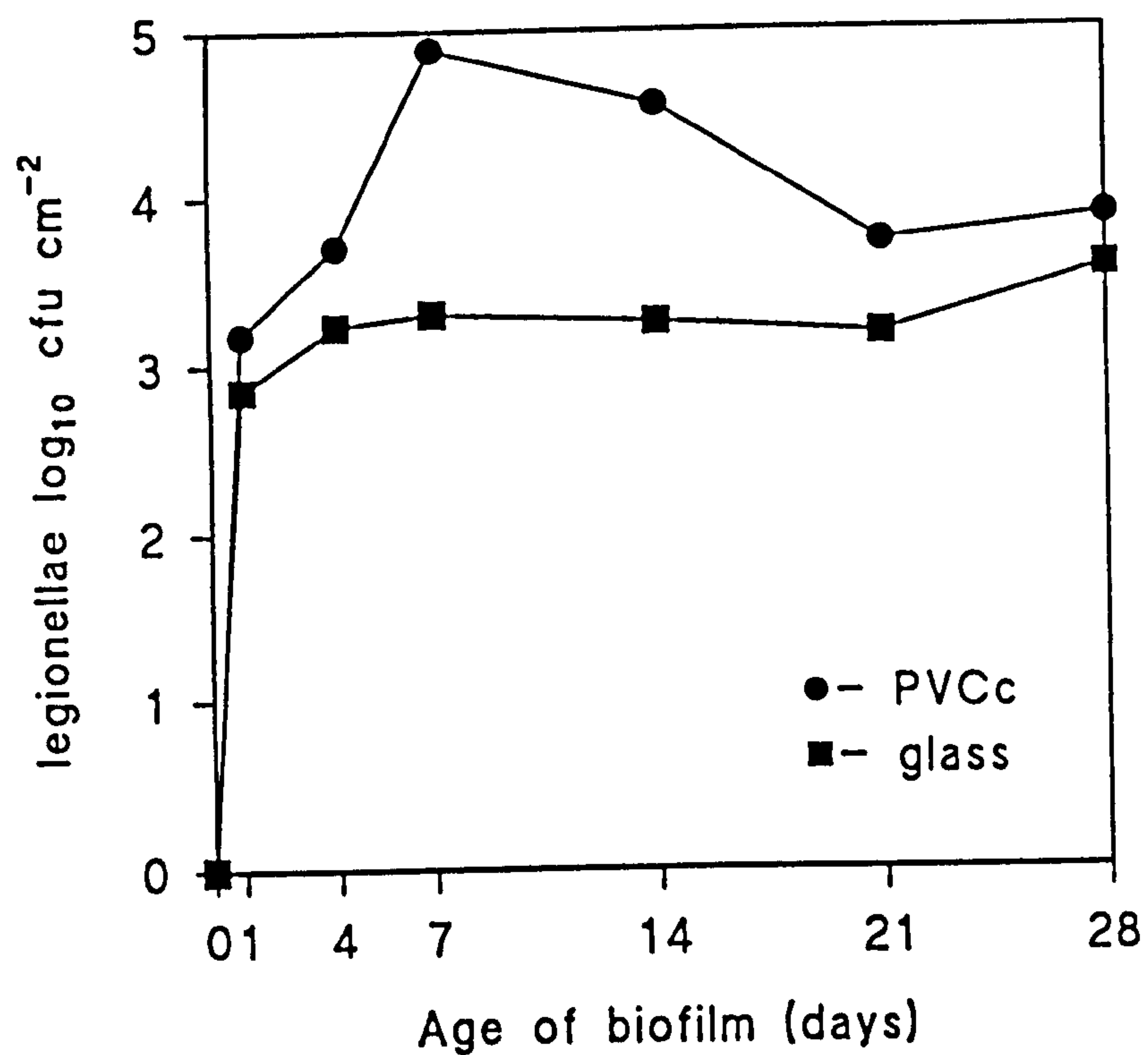


Figure 4.12 Colonisation by *L. pneumophila* of PVCu and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

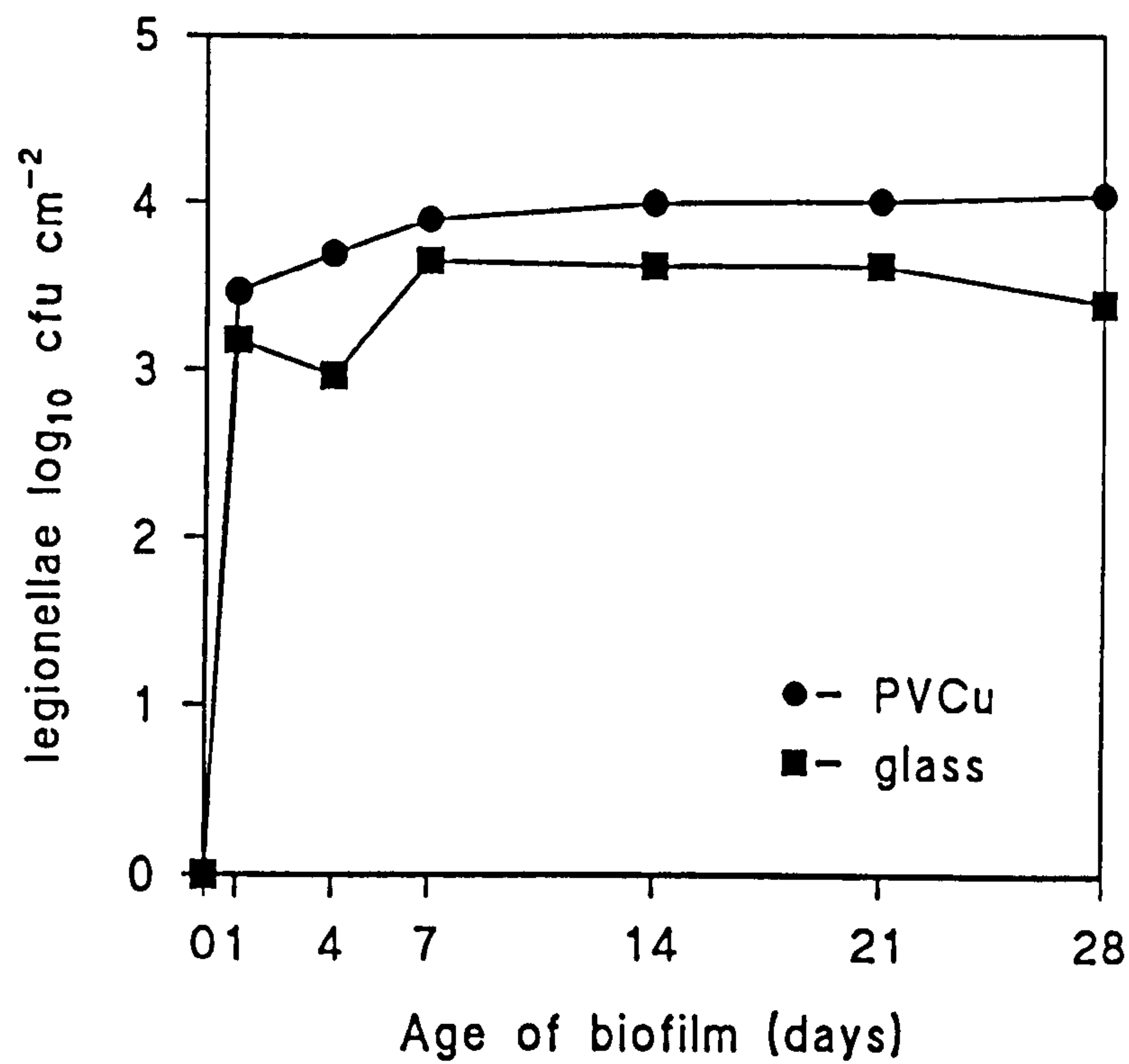


Figure 4.13 Colonisation by *L. pneumophila* of polypropylene and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

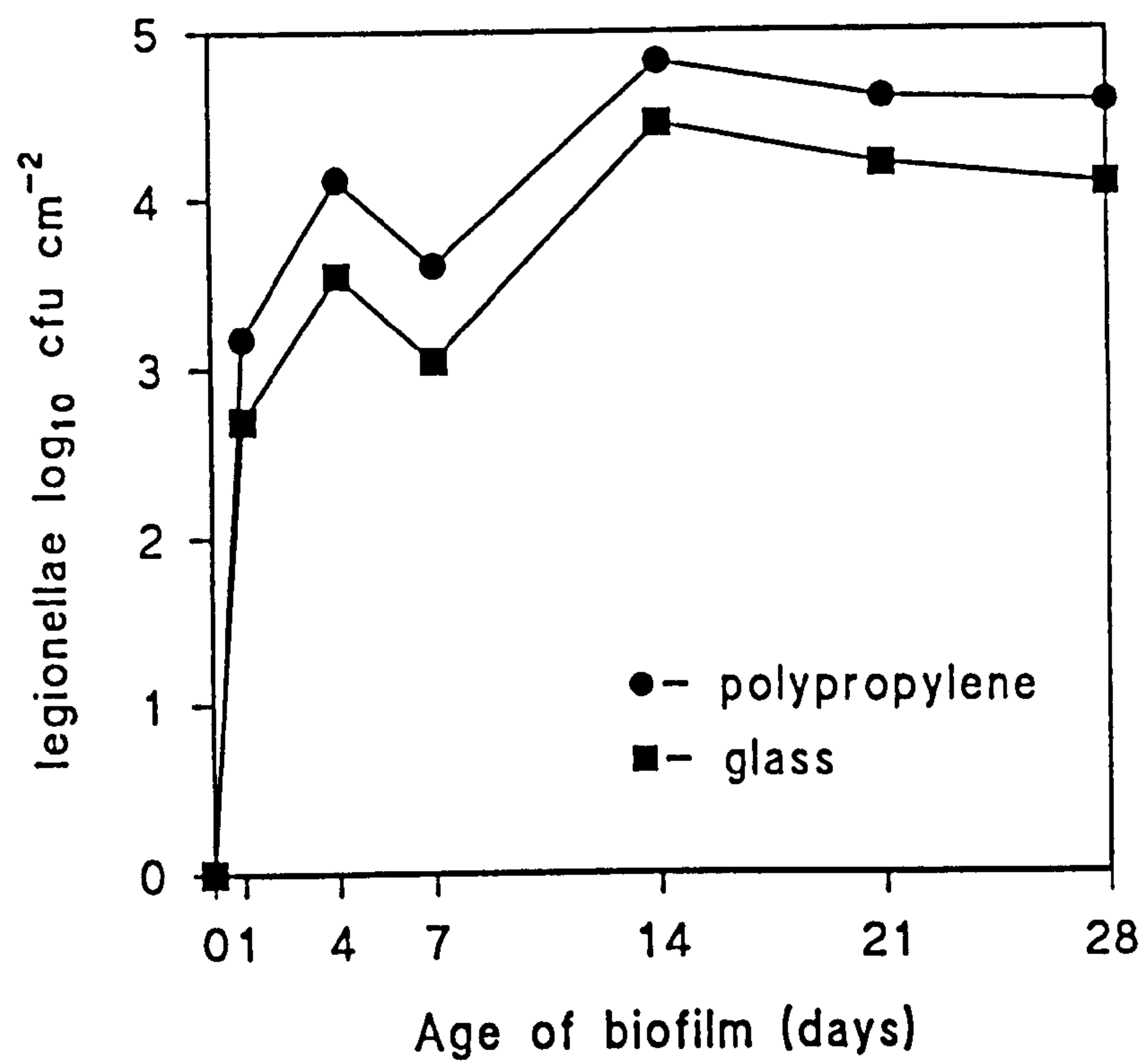


Figure 4.14 Colonisation by *L. pneumophila* of polyethylene and control glass surfaces at 30°C in tap water containing a complex consortia of microorganisms.

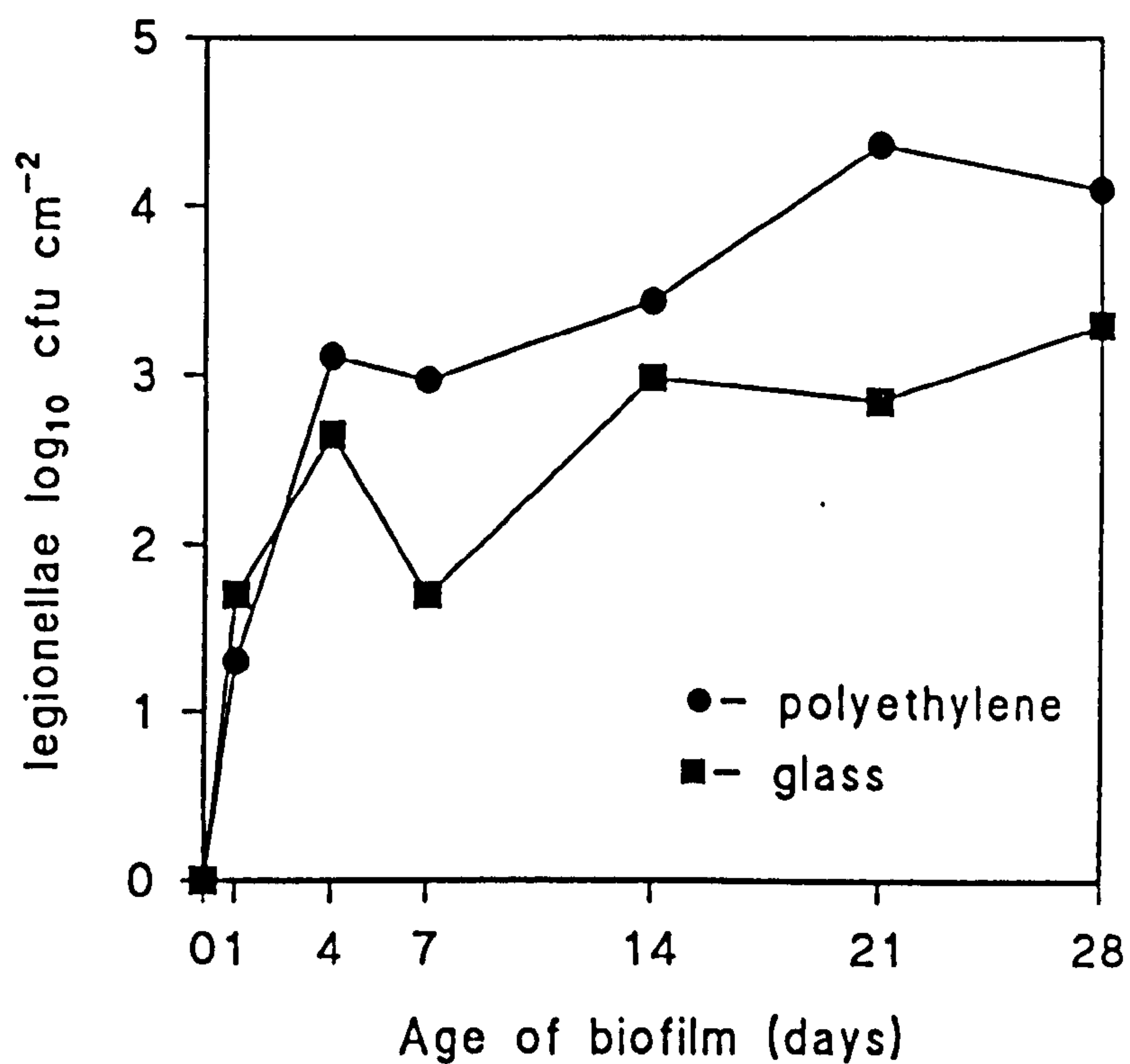


Figure 4.15 Colonisation by *L. pneumophila* of mild steel at 30°C in tap water containing a complex consortia of microorganisms. (ND denotes not done).

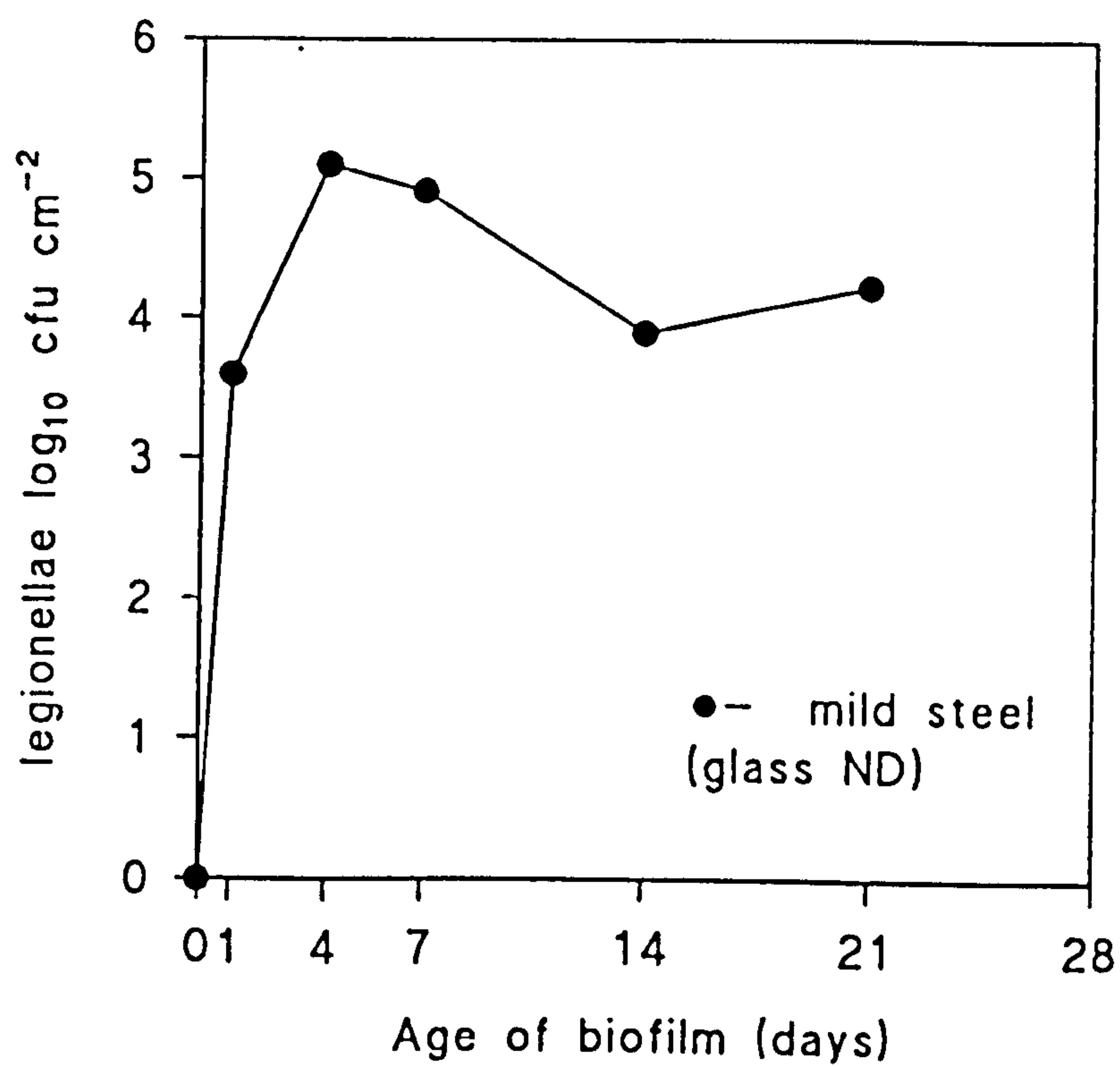
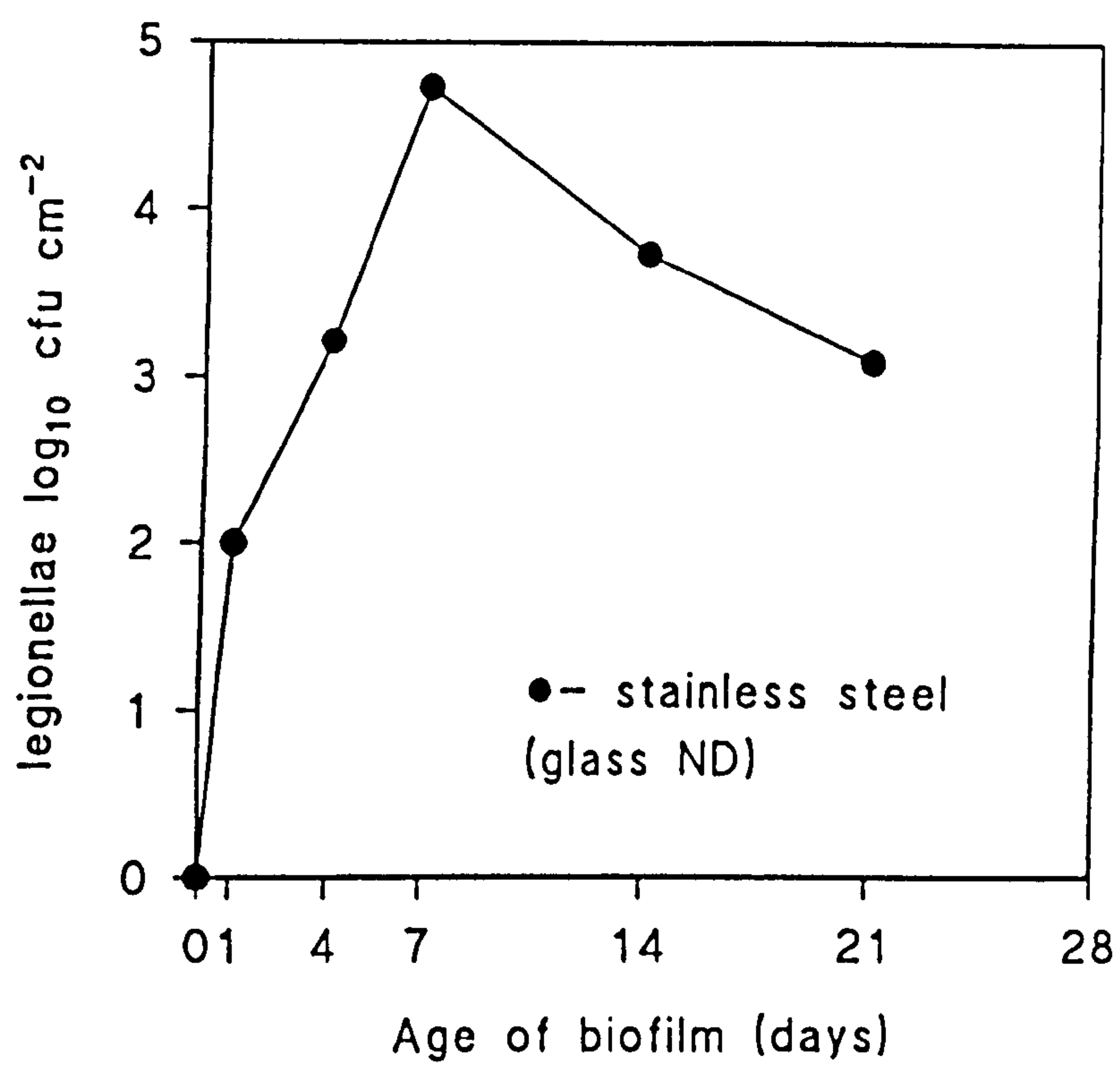


Figure 4.16 Colonisation by *L. pneumophila* of stainless steel at 30°C in tap water containing a complex consortia of microorganisms. (ND denotes not done).



4.3.4 Pioneering microorganisms in biofilms

The microorganisms that colonised the surfaces of the materials surfaces varied in species composition (Table 4.3). Pioneering species (those identified after 24h incubation) on the mild steel were dominated by pseudomonads which accounted for 52% of the flora, the principal species were *P. testosteroni* and *P. paucimobilis*. Other organisms that occurred in high numbers included *Methylobacterium*, *Acinetobacter* and *Klebsiella* spp. The pseudomonads accounted for 61% of the pioneers of the stainless steel surface, with *P. paucimobilis*, *testosteroni*, *P. stutzeri* and *P. vesicularis* being present. The single most abundant microorganism was *Acinetobacter* sp. with 2.6×10^4 cfu cm⁻².

A diverse range of pseudomonads were pioneers on the PVCc surface, including *P. acidovorans*, *P. mendocina*, *P. paucimobilis*, *P. stutzeri* and *P. xylesoxidans*. Actinomycetes were also present. Initial colonisation of the polyethylene was also predominantly by pseudomonads, principal pioneers were *P. fluorescens*, *P. acidovorans* and *Acinetobacter* sp. In contrast, pseudomonads and the other gram negative microorganisms occurred in approximately equal proportions in the biofilm forming on the polypropylene. The most abundant organisms being *P. diminuta* and *Acinetobacter* sp. With *P. fluorescens*, *P. mendocina*, *Alcaligenes* sp, *Flavobacterium* sp., *Methylobacterium* sp. and actinomycetes representing a small proportion of the biofilm flora. Biofilms developing on the PVCu surface were composed of a mixture of gram negative microorganisms (principally *P. acidovorans* and *P. vesicularis*) and by actinomycetes.

The latex surface was initially colonised by a mixed flora, including *P. xylesoxidans*, *Acinetobacter* sp. and actinomycetes. Primary colonisation of ethylene-propylene was dominated by *Acinetobacter*, *Aeromonas*, *Flavobacterium* and *Alcaligenes* spp.

Table 4.3 Pioneering microorganisms present after 24h incubation on the surface of the various plumbing materials (cfu cm⁻² x10³).

	mild steel	stainless steel	latex	ethylene-propylene	polypropylene	polyethylene	PVCu	PVCc
<i>L. pneumophila</i>	3.95	0.1	15	9	1.5	0.5	2.9	1.5
<i>P. aeruginosa</i>					1.9	260		
<i>P. acidovorans</i>							37	880
<i>P. diminuta</i>				1000	22			
<i>P. fluorescens</i>					26	380	1	
<i>P. maltophilia</i>	80	10	8000	100		20	3	
<i>P. mendocina</i>	60	25	4000	3	3		4.7	210
<i>P. paucimobilis</i>	100	10		1000		0.2	1.2	300
<i>P. stutzeri</i>	90	1						
<i>P. testosteroni</i>	410	12	9000		1	80	2	150
<i>P. vesicularis</i>						6.2	59	
<i>P. xyloxydans</i>			36000					310
<i>Actinomyces</i>	10	6	34000		1		19	200
<i>Aeromonas</i>				8000				
<i>Alcaligenes</i>	70			2000	4	0.2		
<i>Flavobacterium</i>	40	7	800	1000	3	0.3	0.2	290
<i>Methylobacterium</i>	110	4			3		5	
<i>Klebsiella</i>	130							
<i>Acinetobacter</i>	300	26	40000	10000	17	440	0.6	

Numbers of non-legionella populations are represented as a sum of those cfu cm⁻² occurring on R2A, BCYE and GVPC media.

Approximately 15% of the biofilm was comprised of pseudomonads, with *P. diminuta* and *P. maltophilia* occurring in equal numbers.

4.3.5 Climax communities in biofilms

The climax community was determined by investigating the microbial population present after 21 days on each of the surfaces. Pseudomonads were dominant in the climax community on the mild steel surfaces, representing 62% of the total flora (Table 4.4). *P. aeruginosa* and *P. vesicularis* succeeded *P. mendocina*, *P. testosteroni* and *Klebsiella* sp. A similar proportion of pseudomonads were present on the surface of stainless steel where *Aspergillus* and *Alcaligenes* sp. replaced *Flavobacterium* sp. and *P. mendocina* within the community.

The diversity of microorganisms within the biofilm on latex was increased by the addition of *P. paucimobilis* and *P. stutzeri* to the surface, however, pseudomonads accounted for only 11% of the flora. A similarly low proportion of the flora on ethylene-propylene was composed of pseudomonads and the climax community included high numbers of *Aspergillus* sp. and actinomycetes. The loss of several pseudomonads and other gram negative microorganisms reduced species diversity on the mature biofilm on the ethylene-propylene.

Pseudomonads composed between 37-49% of the biofilm communities on the plastic surfaces. Several species of pseudomonads were replaced by *P. paucimobilis* and *Acinetobacterium* sp. were succeeded by *Acinetobacter* sp. in the mature biofilm on polypropylene. Similar shifts in the populations of pseudomonads on the surfaces of polyethylene and PVCu, with *P. paucimobilis* becoming the predominant microorganism in the flora, although other species were also present. *Methylobacterium* were also represented in the mature community on polyethylene. In contrast, *P.*

Table 4.4 Climax communities of microorganisms on the surfaces of the various plumbing materials (cfu cm⁻² x10³) after incubation of biofilms for 21 days.

	mild steel	stainless steel	latex	ethylene-propylene	polypropylene	polyethylene	PVCu	PVCc
<i>L. pneumophila</i>	17	13	150	500	37	13	11	7.9
<i>P. aeruginosa</i>	30							
<i>P. acidovorans</i>						40		11
<i>P. diminuta</i>						2		
<i>P. fluorescens</i>				1000				3
<i>P. maltophilia</i>	10	11	3000			10	10	
<i>P. mendocina</i>			13			40	0.01	
<i>P. paucimobilis</i>	30	36	5000	1600	790	170	140	36
<i>P. stutzeri</i>	140	70	2000					0.1
<i>P. testosteronei</i>		180				20		8
<i>P. vesicularis</i>	250							3
<i>P. xyloxydans</i>						7		40
<i>Actinomyces</i>	130	2	7000	8000		9	0.01	2.8
<i>Aeromonas</i>			6000					
<i>Alcaligenes</i>	10	10			320	80		30
<i>Flavobacterium</i>	41		15000	2400		0.2	90	50
<i>Methylobacterium</i>	20	150			140	30	60	23
<i>Klebsiella</i>			31000					
<i>Acinetobacter</i>	70	39	22000	3100	400	180	40	60
<i>Aspergillus</i>		0.2		4400				

Numbers of non-legionella populations are represented as a sum of those cfu cm⁻² occurring on R2A, BCYE and GVPC media.

paucimobilis. *P. testosteroni* and *P. xylesoxidans* were reduced in the biofilms on the PVCc surface with *P. mendocina* being absent. *Alcaligenes*, *Methylobacterium* and *Acinetobacter* sp. became members of the microbial community.

4.3.6 Microscopy of biofilms

LATEX: When viewed under the scanning electron microscope the surface of latex was uneven and pores were visible. After 24h exposure to aquatic microorganisms the biofilm which formed on the latex was so extensive that biofilms were visible without magnification. The photomicrographs (Figure 4.17a) showed that the extracellular matrix of polysaccharide produced after 24h completely covered the material. The depth of the biofilm was demonstrated where holes were evident (Figure 4.17b). The microbial flora were embedded within the biofilm matrix and as the biofilm matured the matrix was recolonised by microorganisms (Figure 4.17c). Cracks were evident within biofilms viewed under the scanning electron microscope (Figure 4.17d).

ETHYLENE-PROPYLENE: The biofilm which formed on the copolymer was visible without magnification and the scanning electron micrographs showed that there was considerable depth to the biofilm structure (Figure 4.18b). Microorganisms could be observed embedded within the extracellular matrix and there were large cracks in the biofilm (Figure 4.18a). The 4 day biofilm on the copolymer could be observed embedded within extracellular matrix and there was evidence of amoebae grazing the bacterial flora (Figure 4.18c). As the biofilm continued to develop the microorganisms could be observed to recolonise the surface layers of the extracellular polymer to form biofilms of increasing thickness (Figure 4.18d).

POLYPROPYLENE: After 24h immersion in the chemostat the polypropylene surface had been colonised in localised places by a diverse range of microorganisms (Figure

4.19a). These microcolonies were dispersed over the surface with areas between being virtually uncolonised. As the tile was incubated further, the microcolonies developed in size, particularly well in crevices perhaps due to protection from shear (Figure 4.19b). After 14 days the biofilm covering the plastic was more extensive (Figure 4.19c) and by 21 days more of the surface was covered (Figure 4.21d). Extracellular polymer was evident in the thicker areas between the long chains of rods on the surface.

POLYETHYLENE: The scanning electron micrographs showed that after 24h exposure to the chemostat culture the whole surface of the polyethylene was colonised by a thin layer of microorganisms (Figure 4.20a). Small dense regions of biofilm appeared to accumulate in crevices in the plastic surface (Figure 4.21b). The majority of the microorganisms were rod shaped (Figure 4.20c). The presence of acinomyces within the biofilm was confirmed by the presence of filamentous microorganisms on the surface (Figure 4.20d).

PVCu: After 24h a monolayer of bacteria covered the surface of the plastic (Figure 4.21a). As the biofilm developed the bacteria were observed in an extracellular matrix (Figure 4.21b). Microcolonies of bacteria with similar morphology indicated that bacterial growth was occurring (Figure 4.21c). Filamentous microorganisms were evident in the mature biofilm and amoebae could be observed to graze the bacterial flora (Figure 4.21d).

PVCc: The scanning electron micrographs showed that the biofilm developed on PVCc after 4 days was limited to a low background of microorganisms with distinct microcolonies of bacteria developed in some areas (Figure 4.22a). Predatory ciliated protozoa, including *Lacrymaria* spp., could be observed grazing the 7 day biofilm on the PVCc (Figure 4.22b) and microcolonies of bacteria were grazed by amoebae. Filamentous organisms were also evident (Figure 4.22c). The mature biofilm was predominantly composed of rod shaped bacteria and chains of bacterial rods (Figure

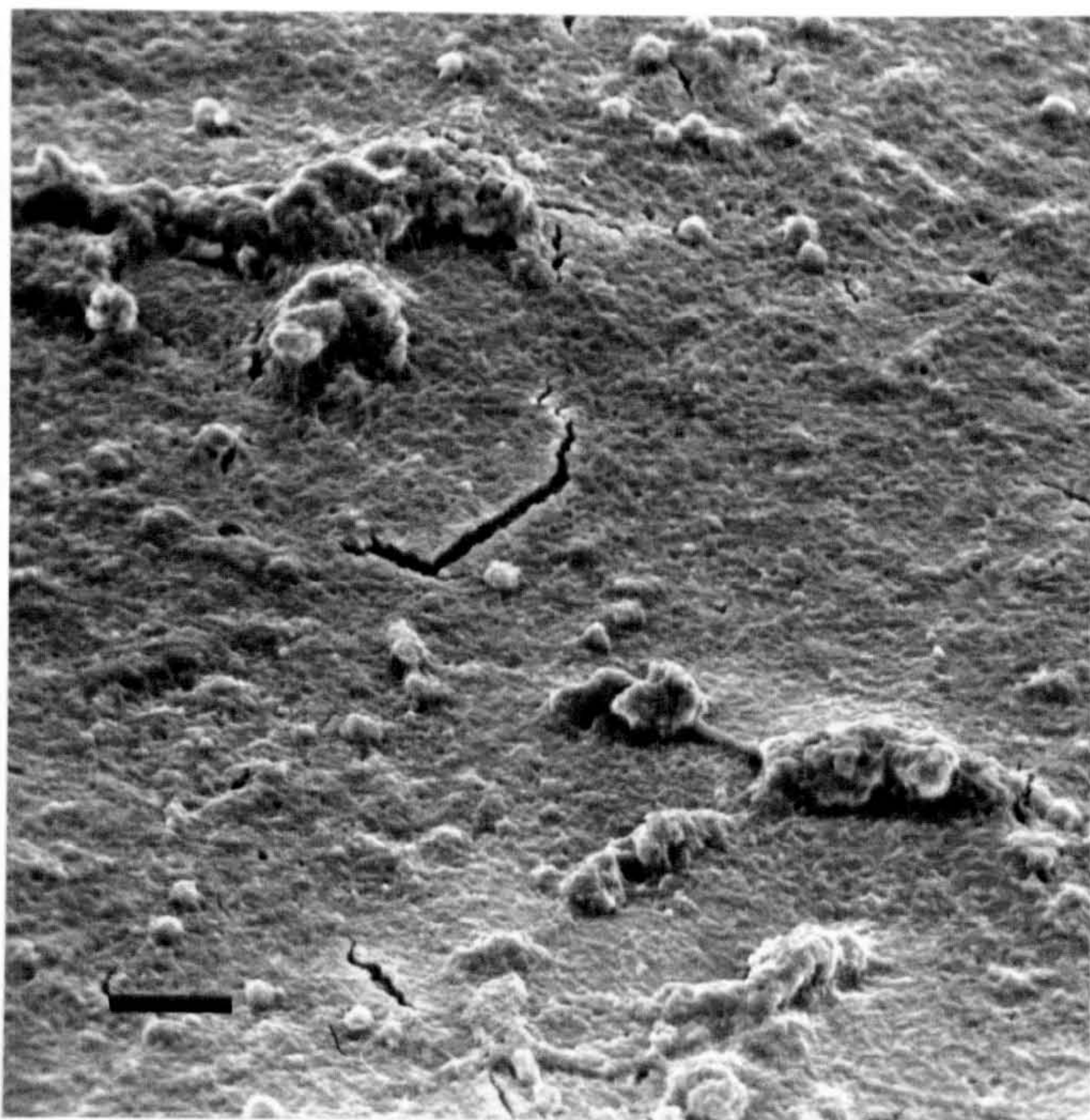
4.22d).

MILD STEEL: The mild steel was rusty after only 24h immersion into the chemostat culture and the scanning electron micrographs show how corroded the surface had become (Figure 4.23a). The surface was completely covered in corrosion products but filamentous organism were evident (Figure 4.23b).

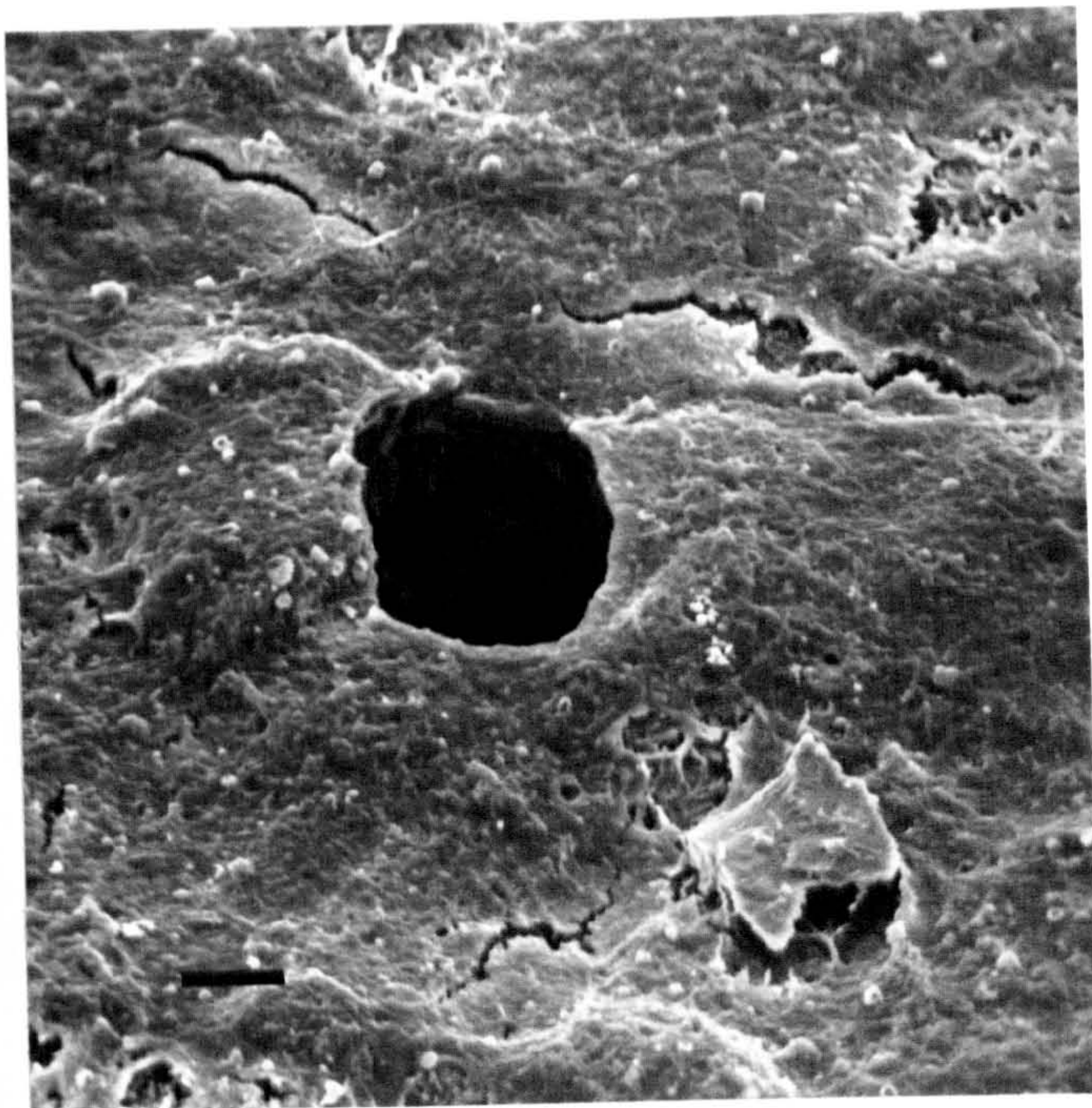
STAINLESS STEEL: The stainless steel surface was also partially covered by corrosion products but to a lesser extent than the mild steel (Figure 4.23c). Microorganisms of similar morphology as those occurring on mild steel were evident (Figure 4.23d)

Figure 4.17 Scanning electron micrographs showing colonisation of the latex surface after 1 (a), 7 (b), 14 (c) and 28 (d) days at 30°C. Bar denote 10 μ m.

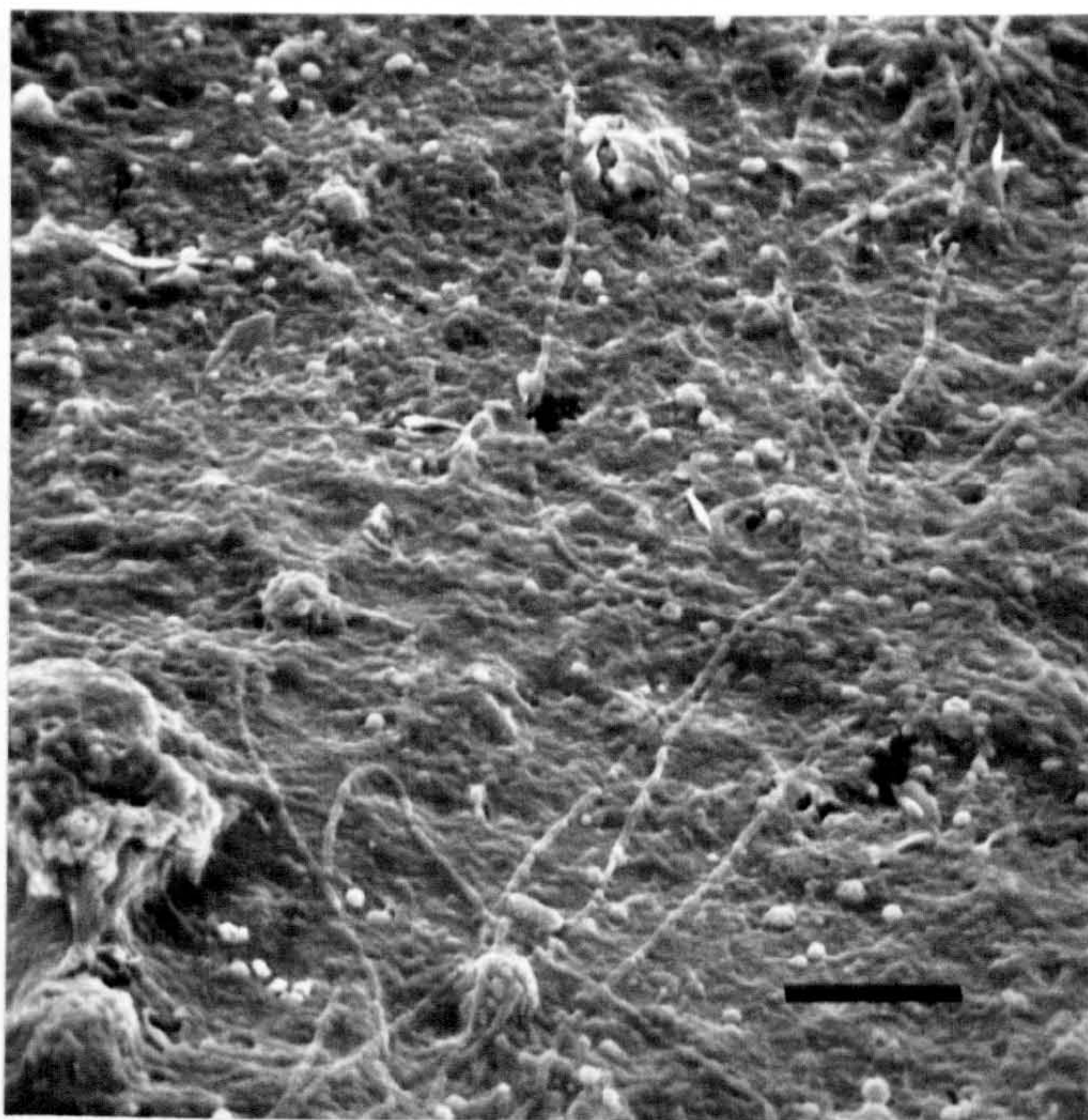
(a)



(b)



(c)



(d)

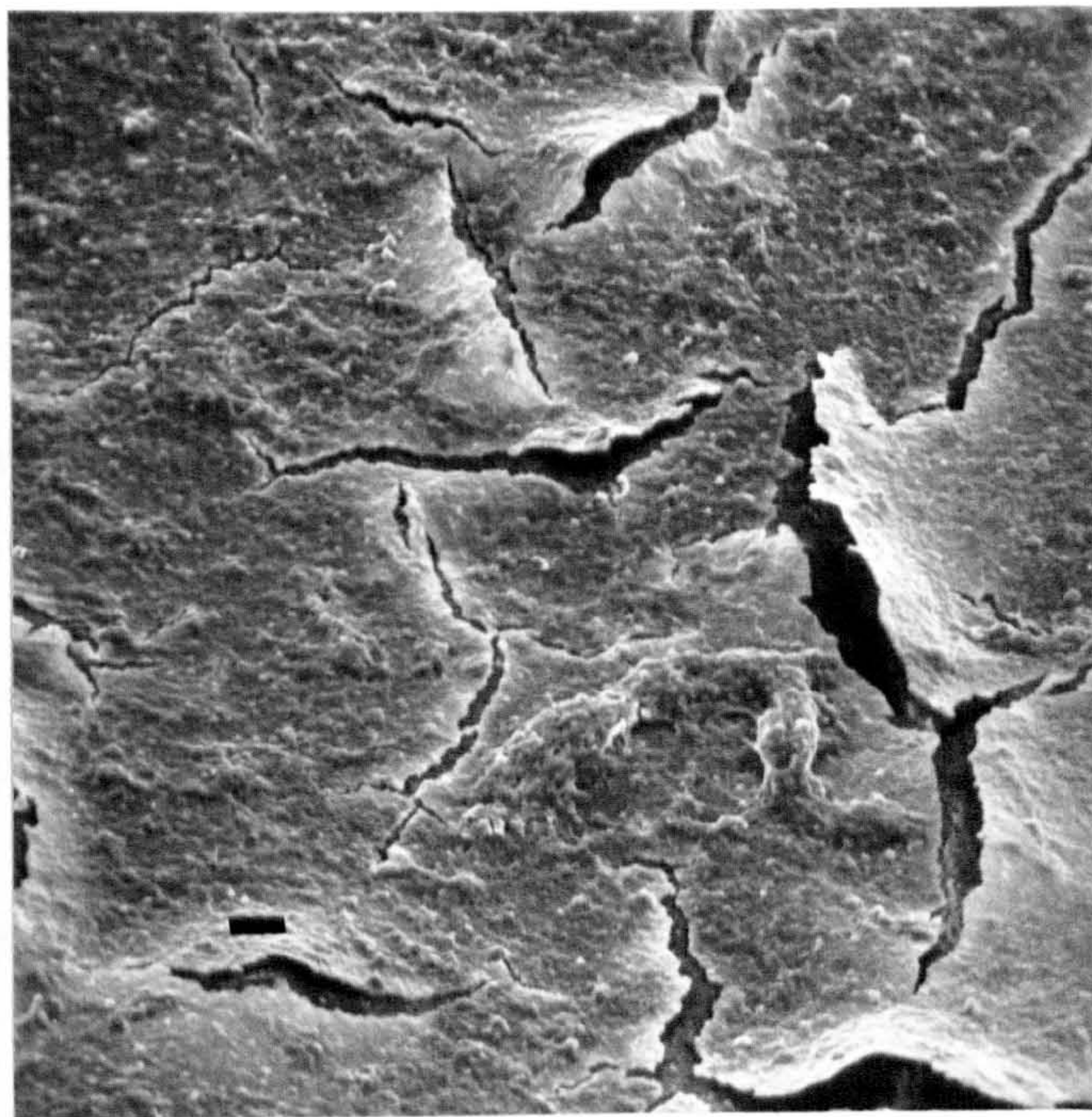
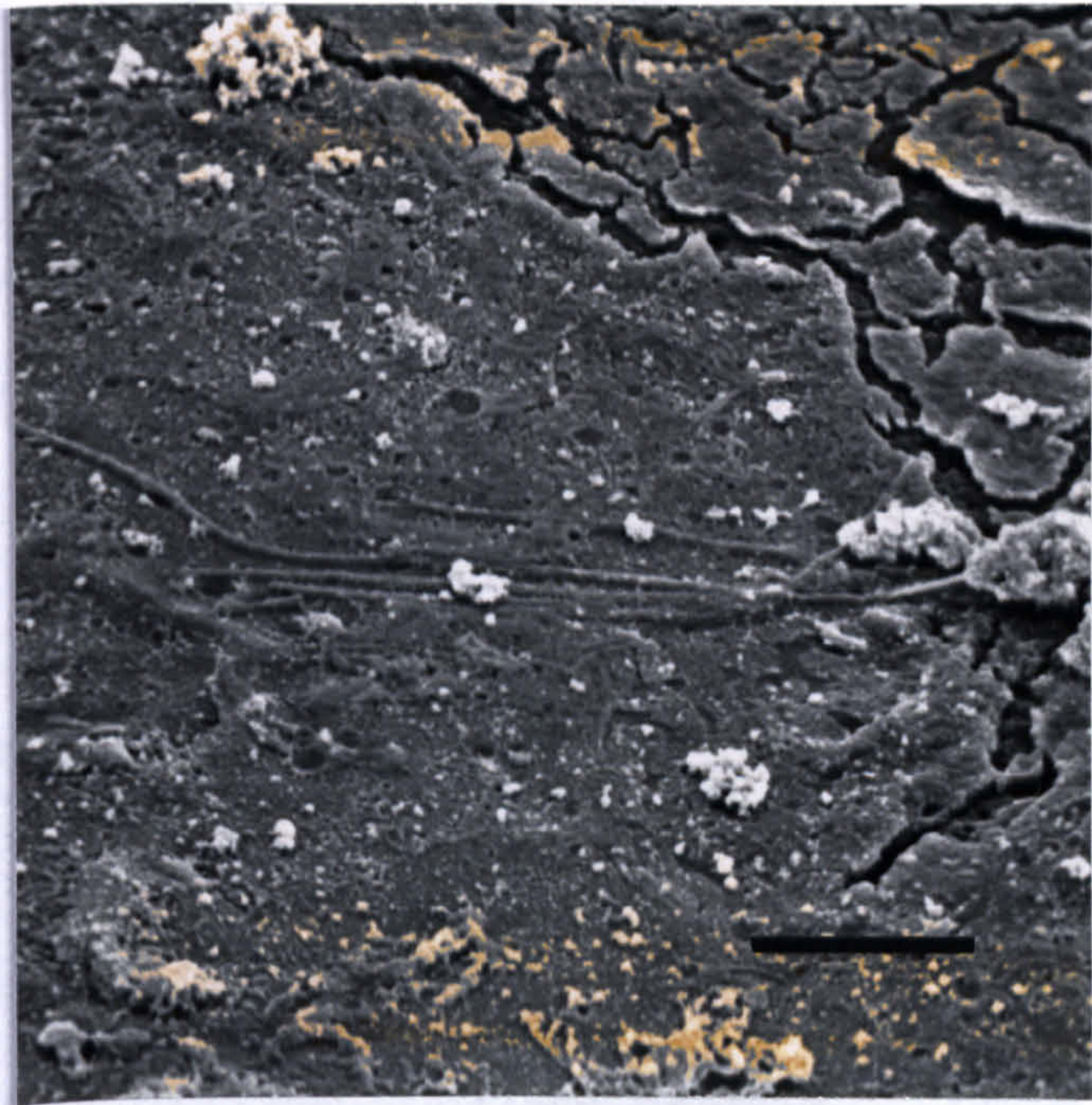
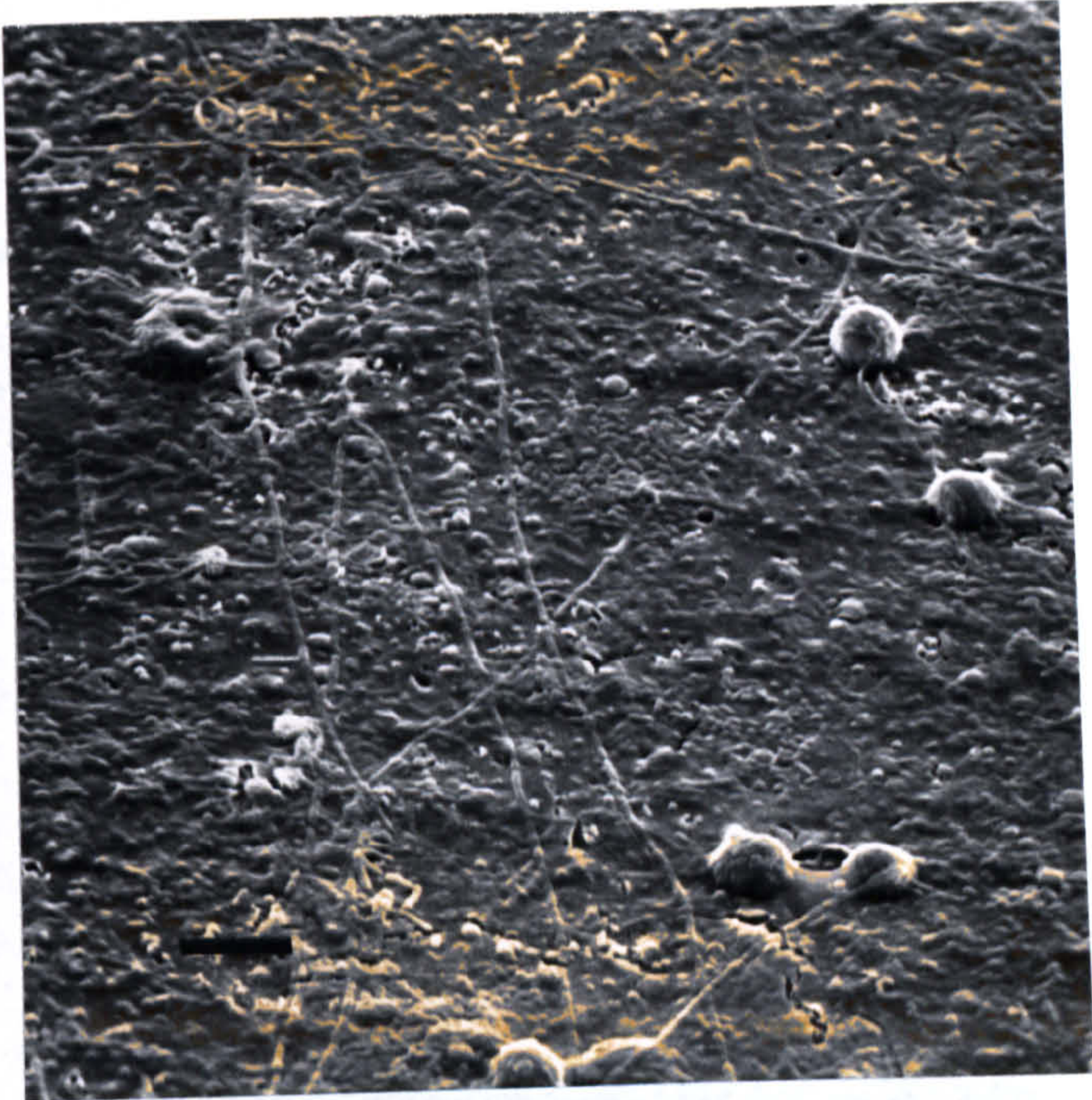


Figure 4.18 Scanning electron micrographs showing the colonisation of ethylene-propylene after 1 (a), 7 (b), 21 (c) and 28 (d) days at 30°C. Bar denotes 10µm.

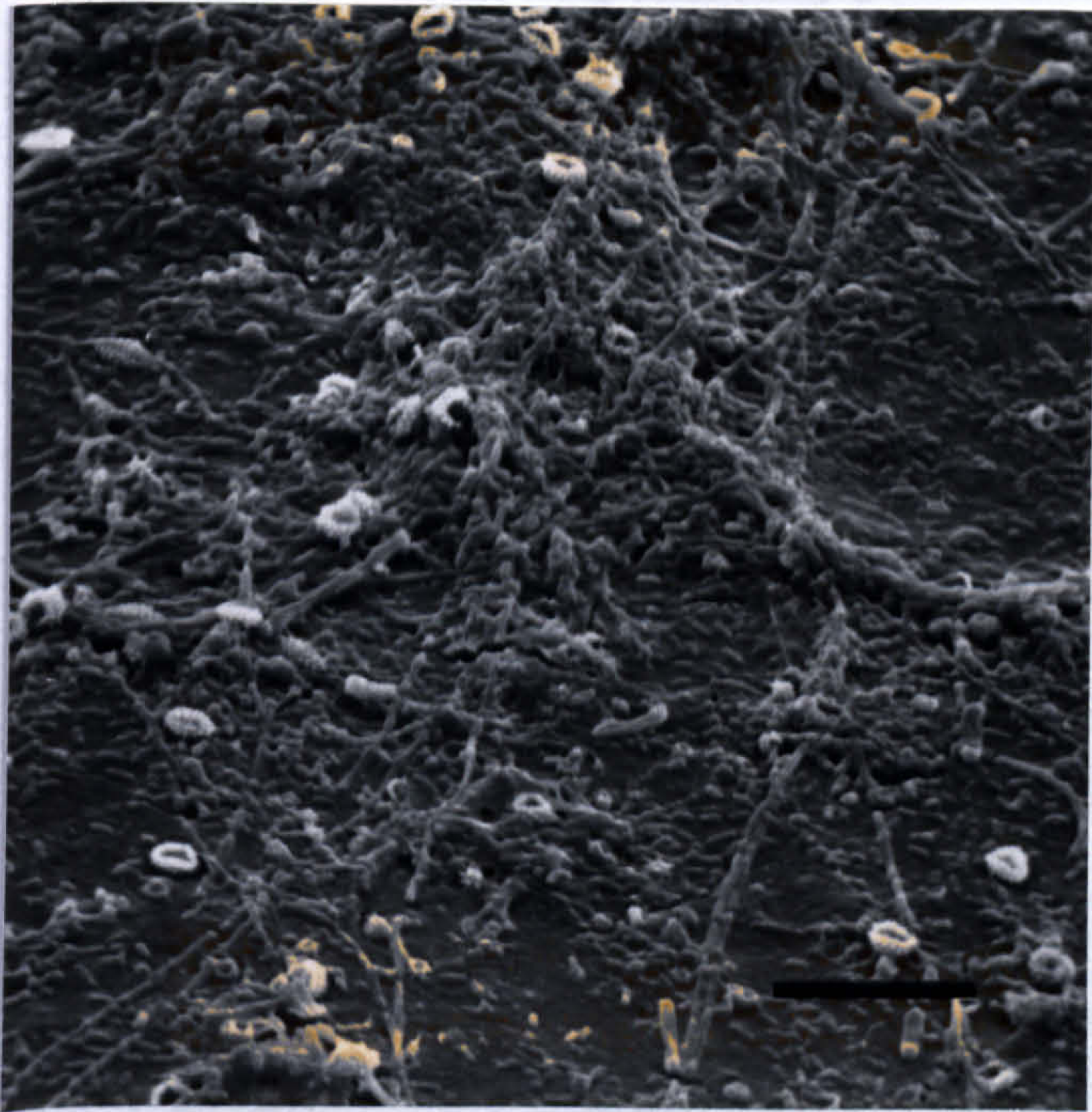
(a)



(b)



(c)



(d)

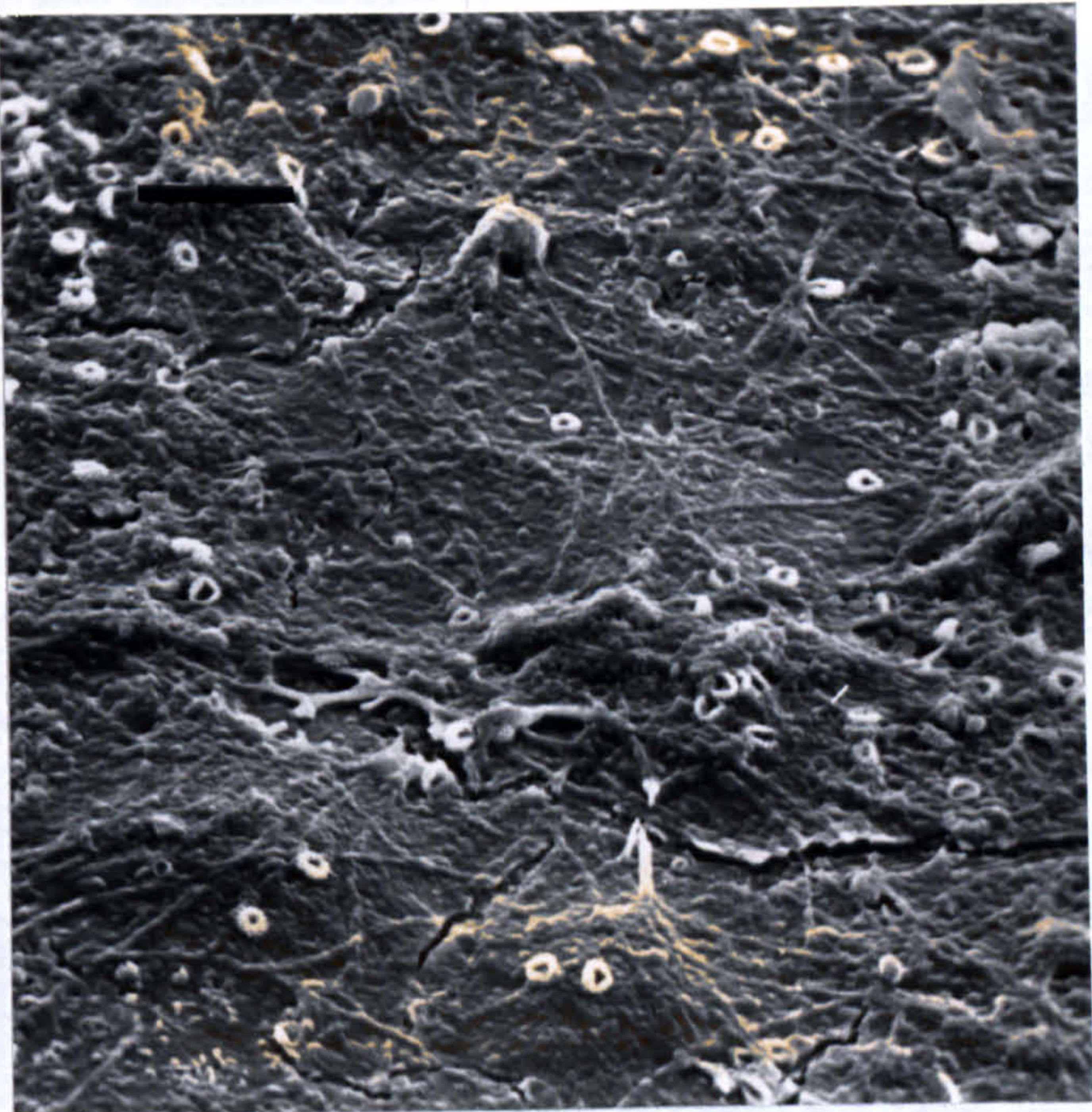
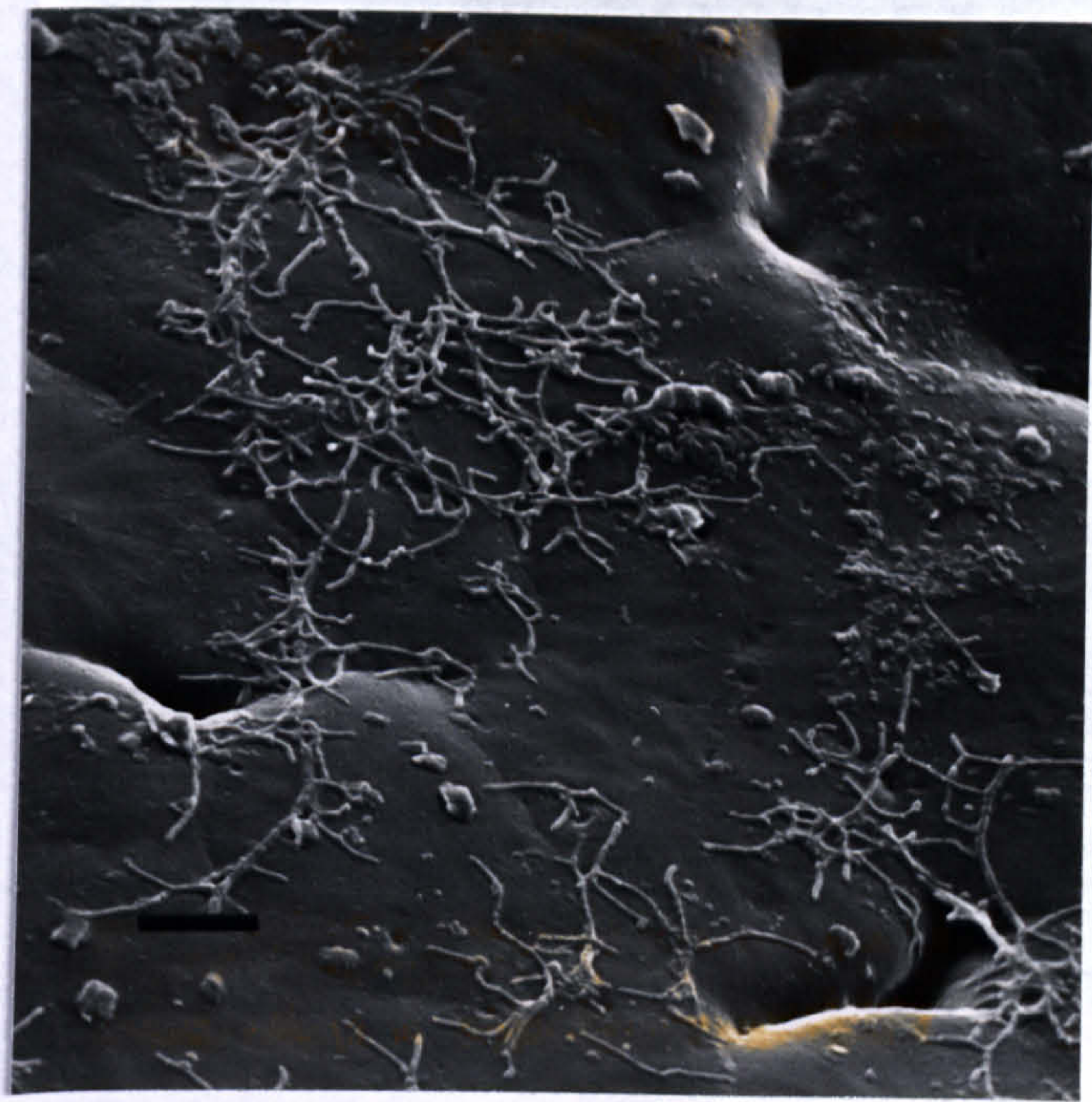
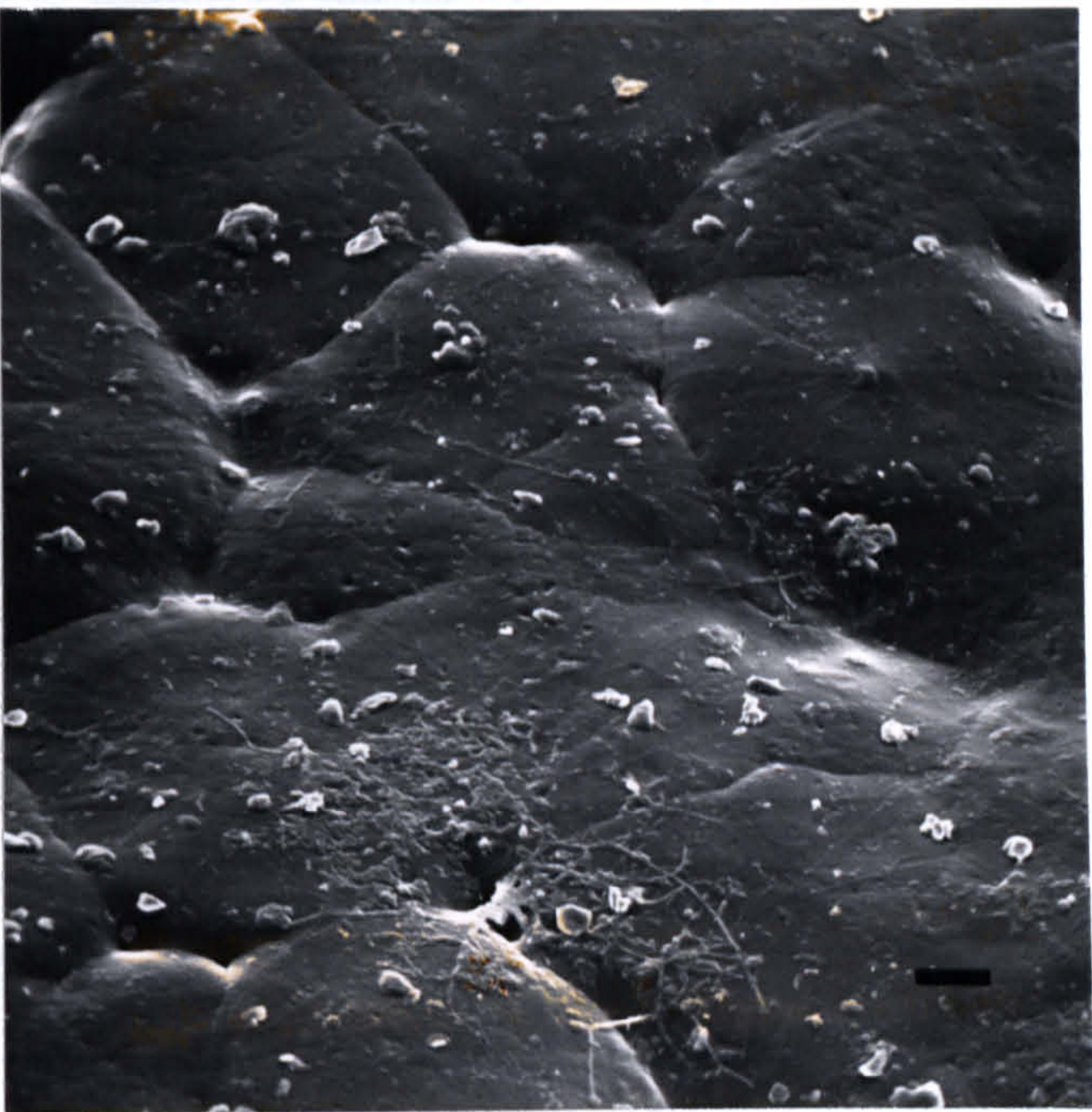


Figure 4.19 Scanning electron micrographs showing the colonisation of polypropylene after 1 (a), 4 (b), 21 (c) and 28 (d) days at 30°C. Bar denotes 10µm.

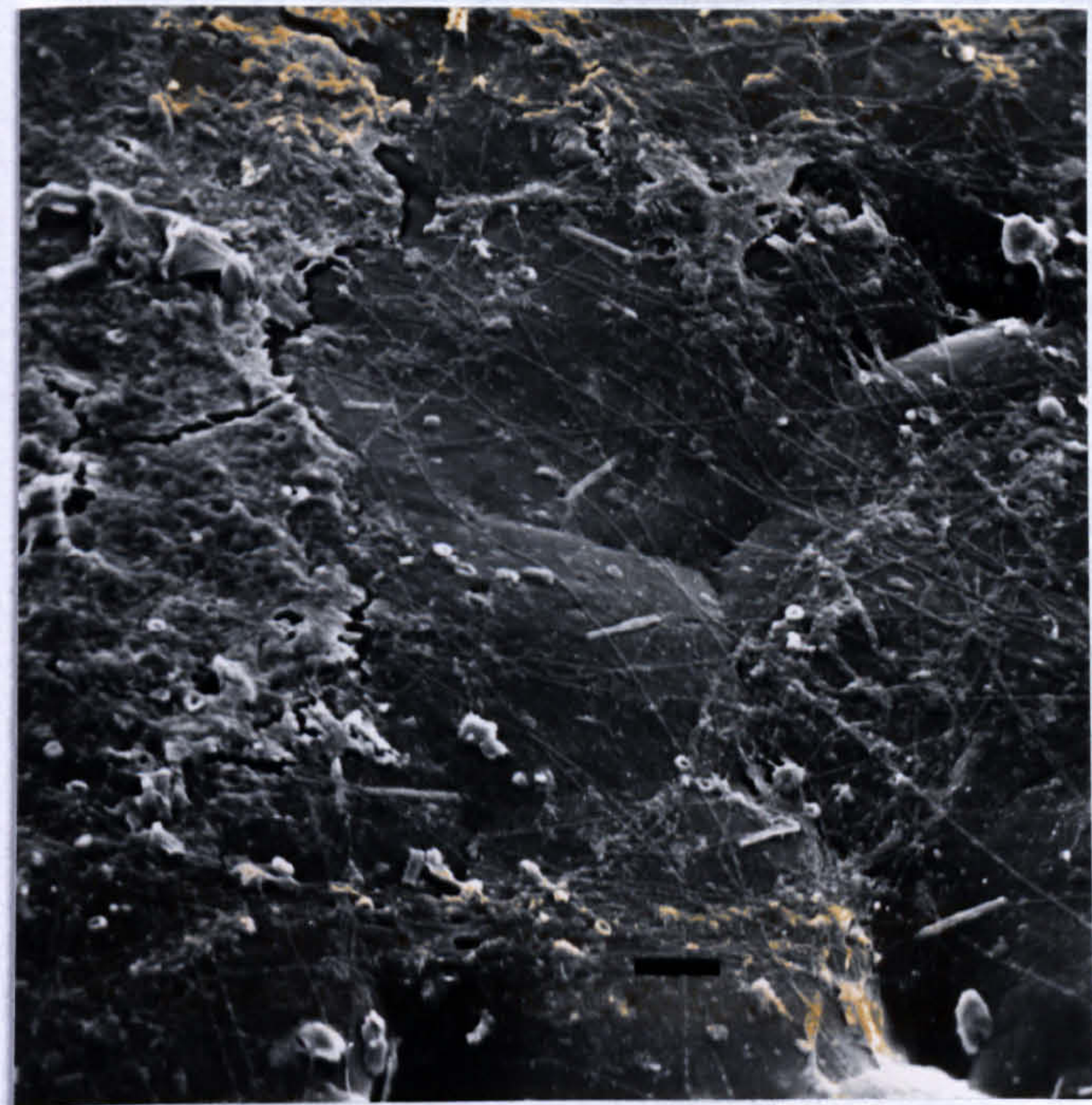
(a)



(b)



(c)



(d)

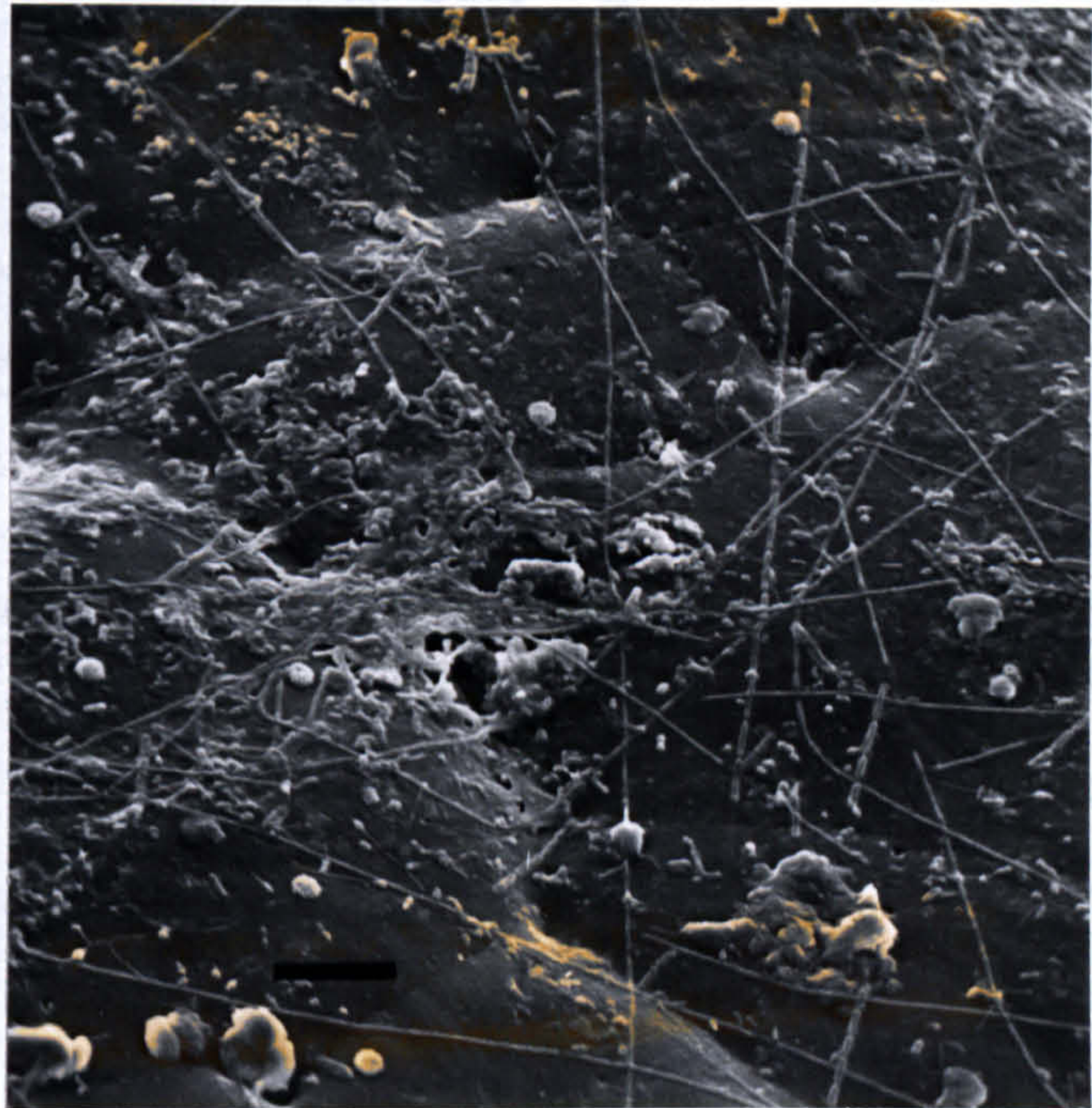
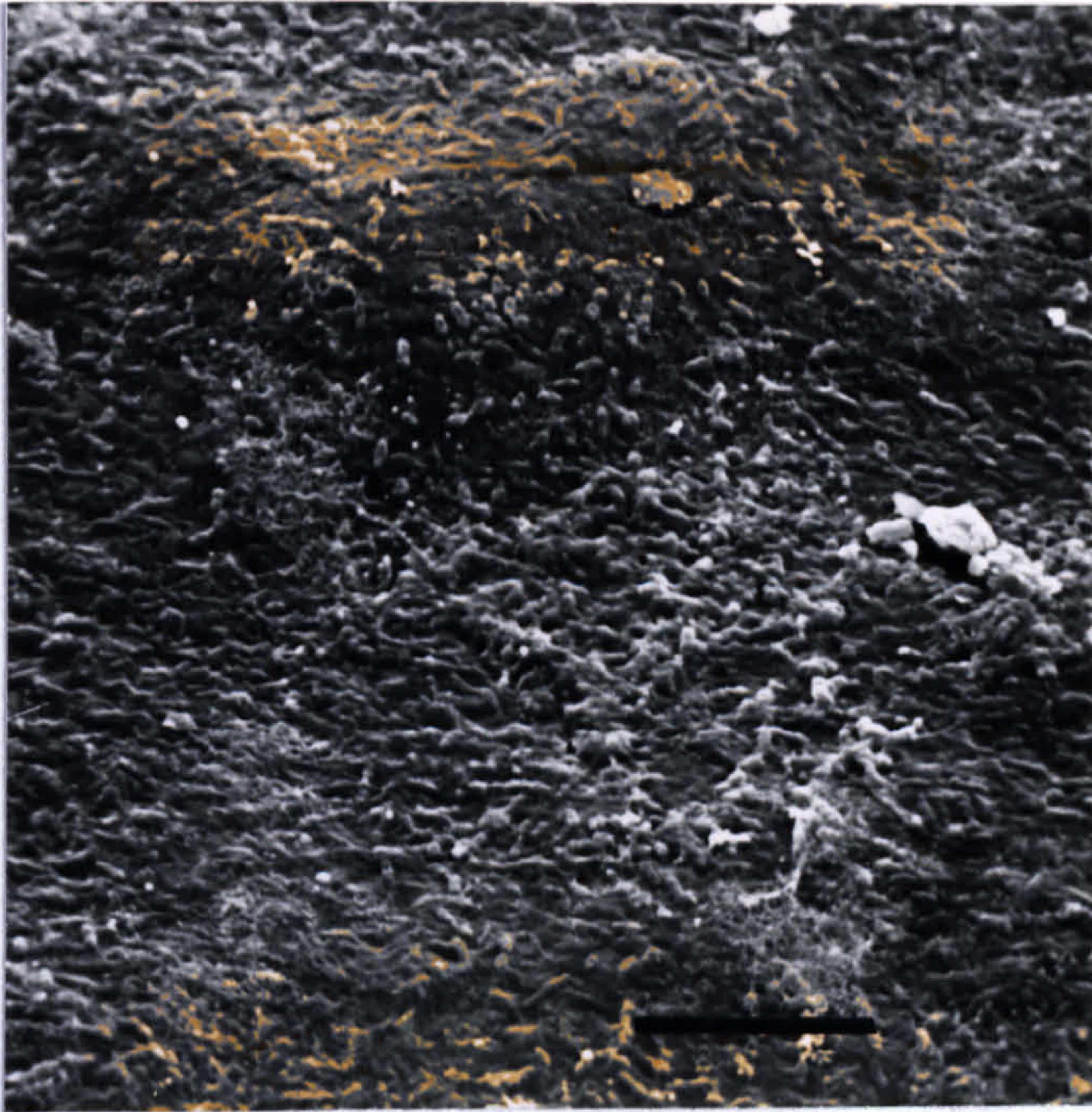
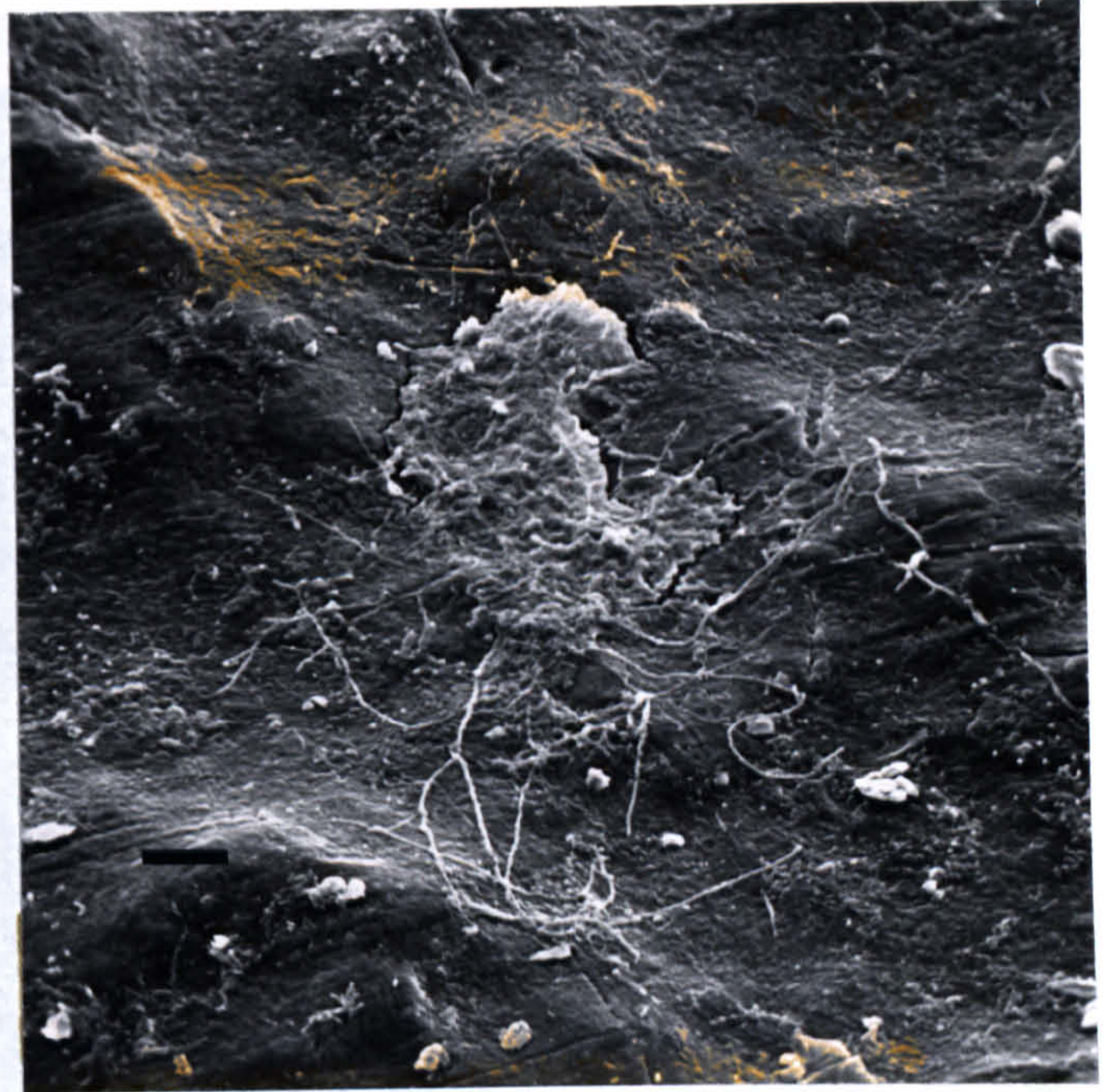


Figure 4.20 Scanning electron micrographs showing the colonisation of polyethylene after 1 (a), 7 (b and c) and 14 (d) days at 30°C. Bar denotes 10µm.

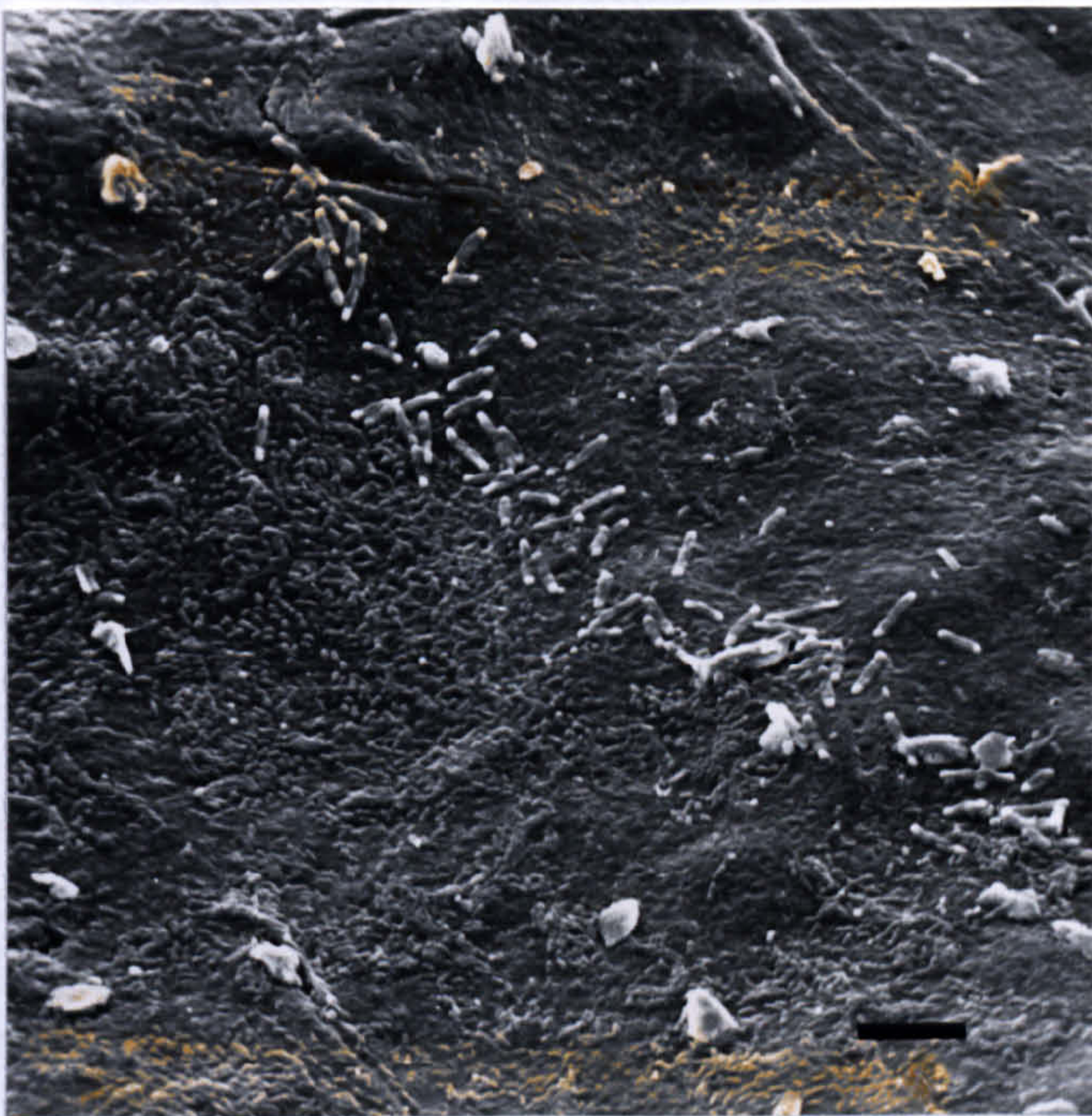
(a)



(b)



(c)



(d)

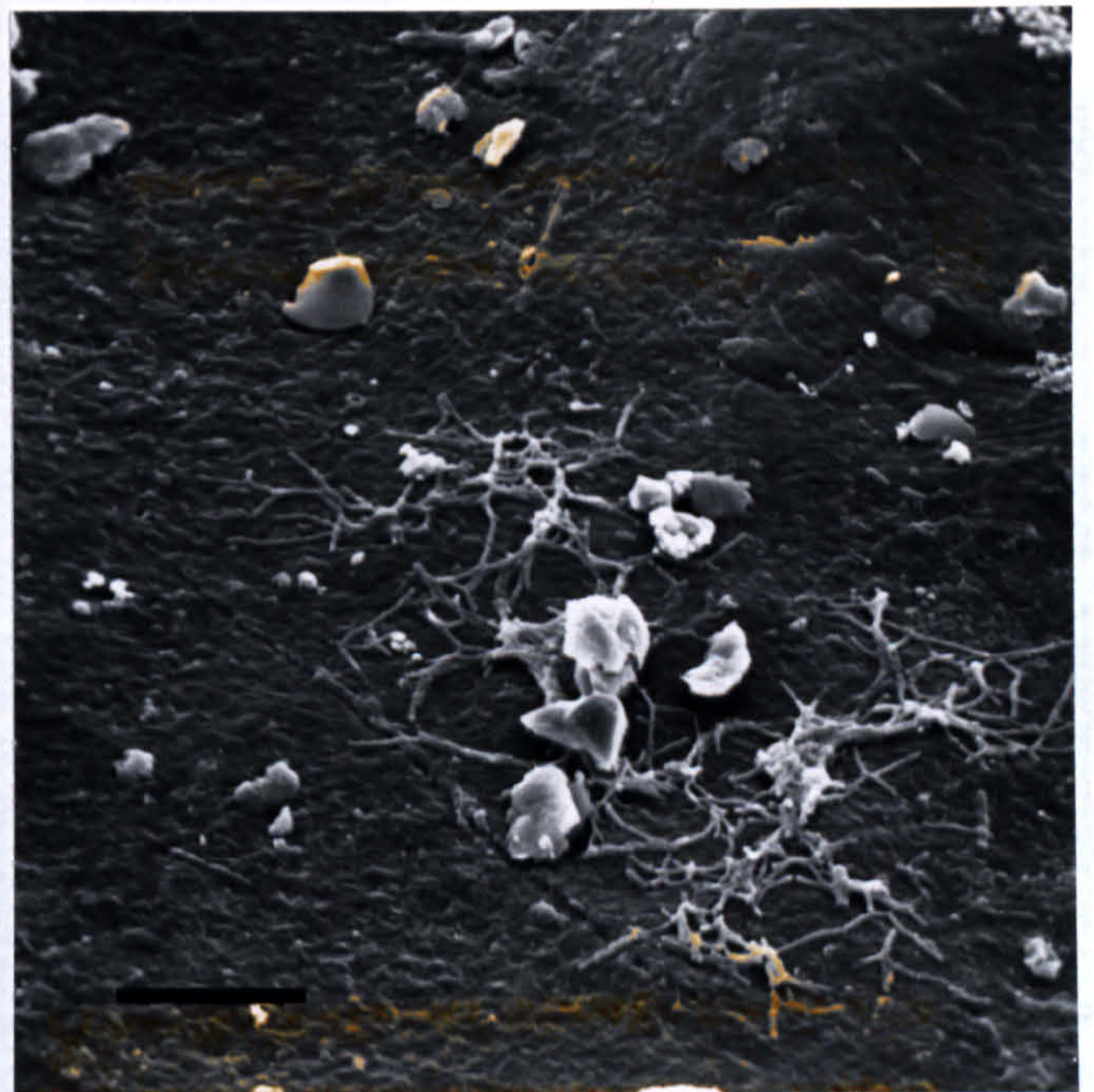


Figure 4.21 Scanning electron micrographs showed the colonisation of PVCu after 1 (a), 7 (b and c) and 14 (d) days at 30°C. Bar denotes 10µm.

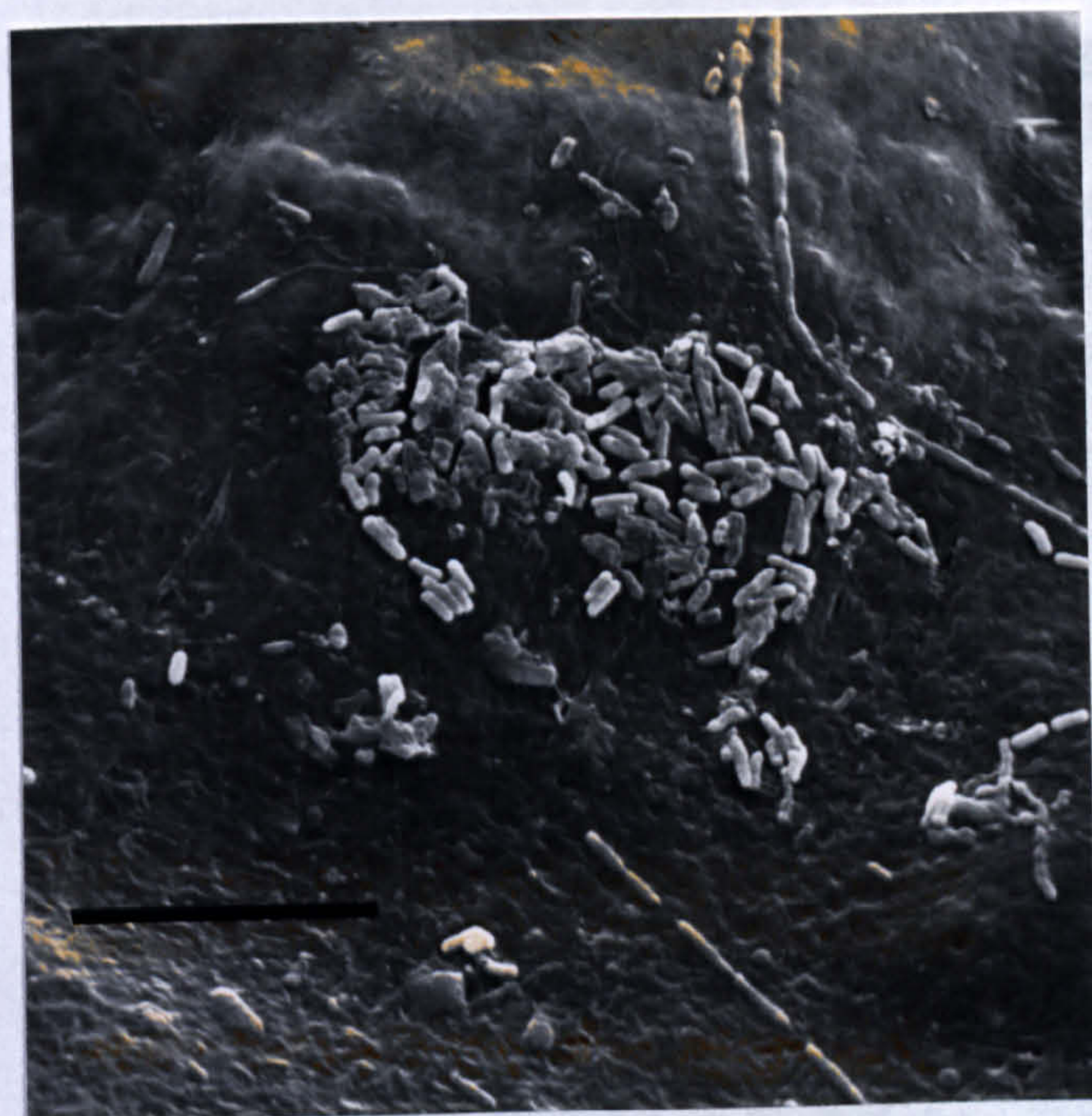
(a)



(b)



(c)



(d)

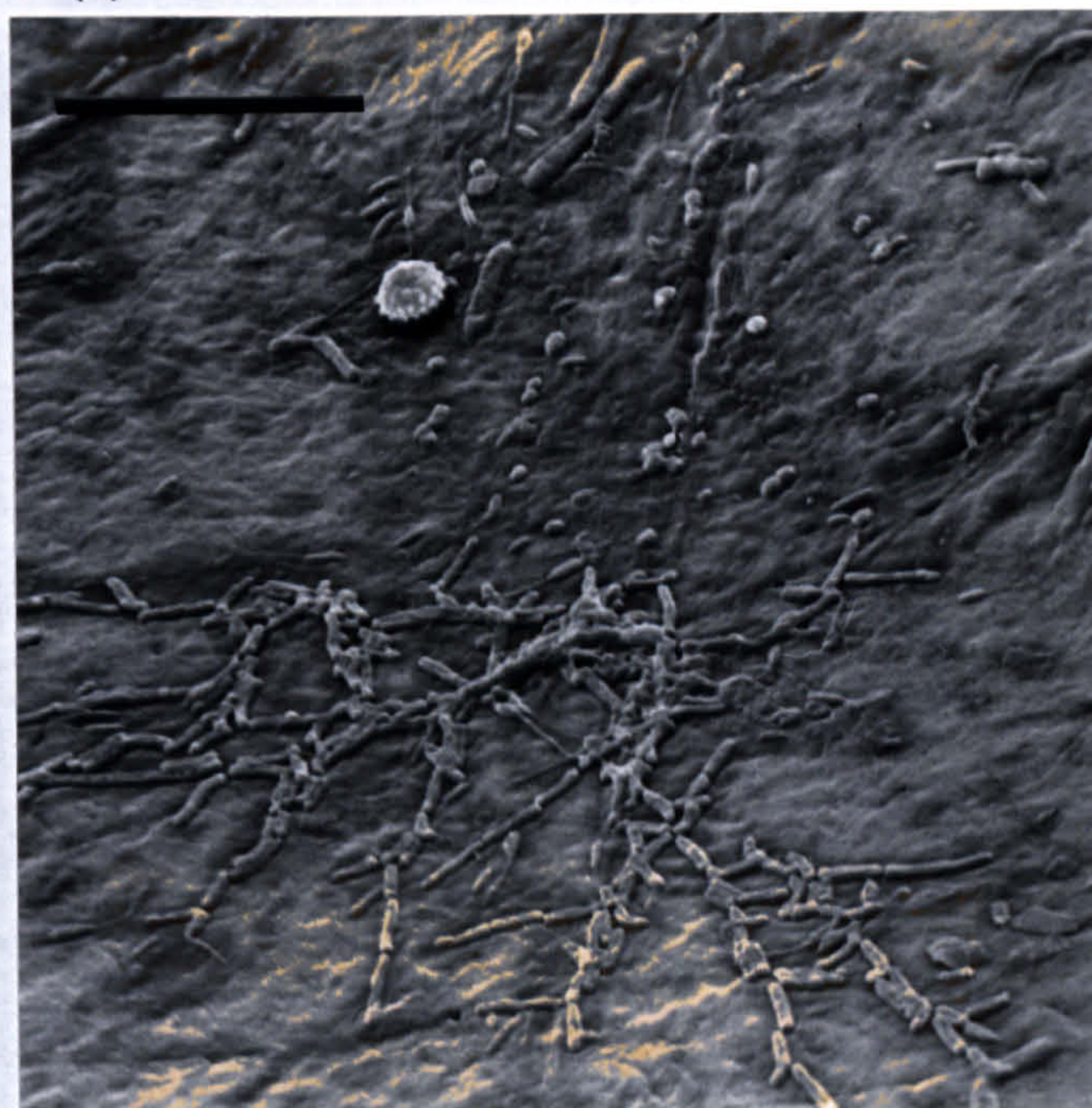
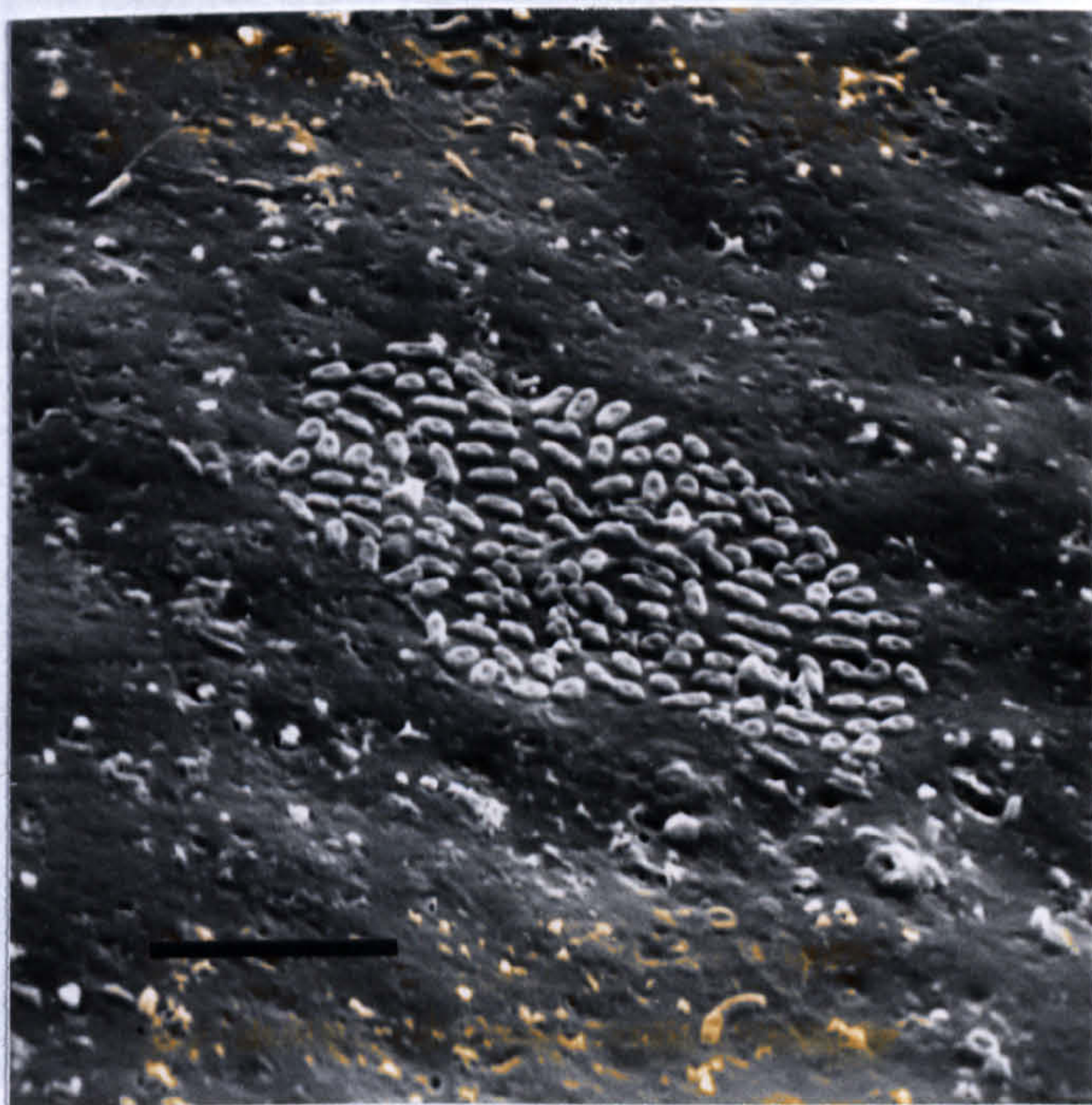
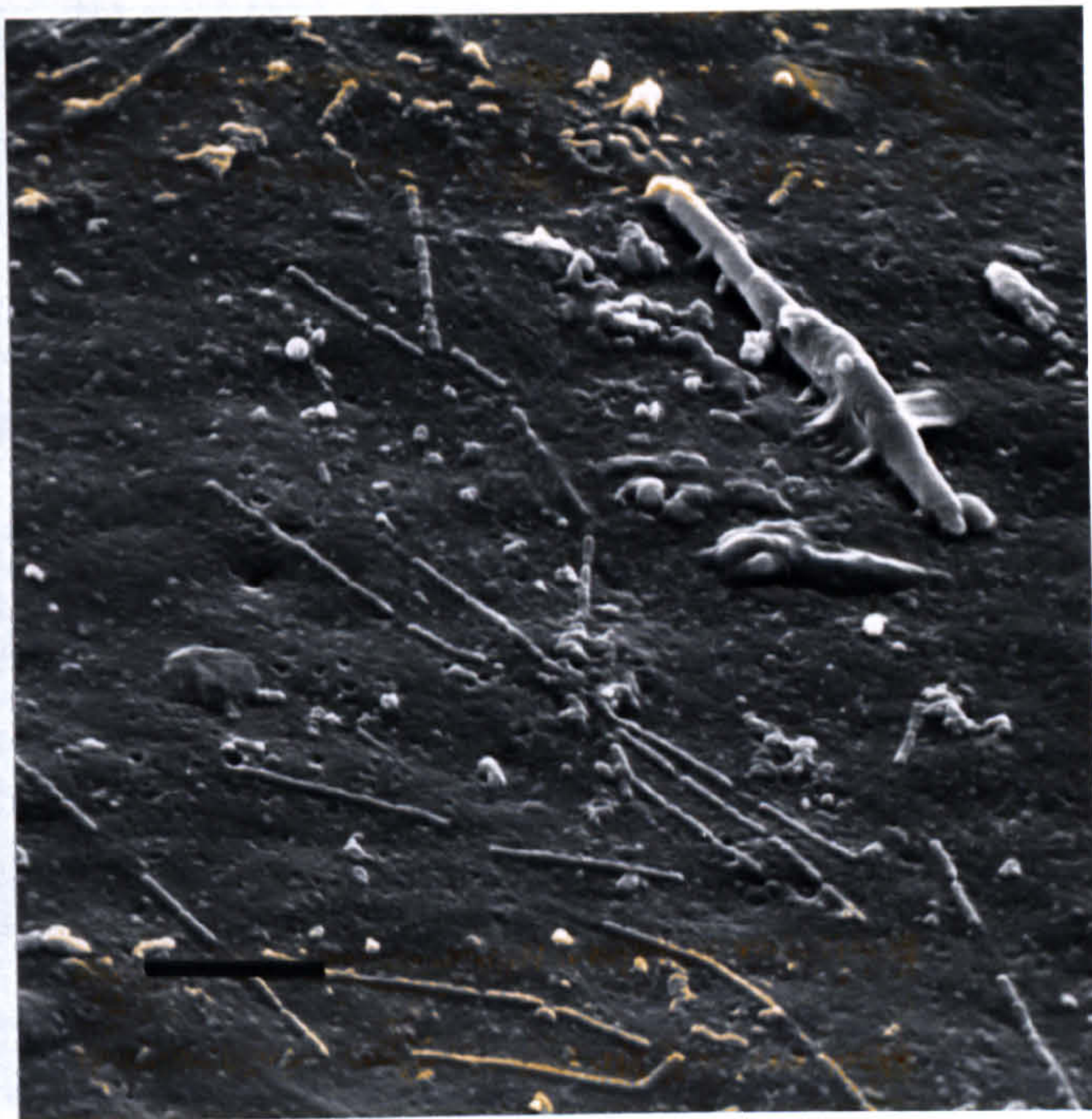


Figure 4.22 Scanning electron micrographs showing the colonisation of PVCc after 4 (a), 7 (b), 21 (c) and 28 (d) at 30°C. Bar denotes 10µm.

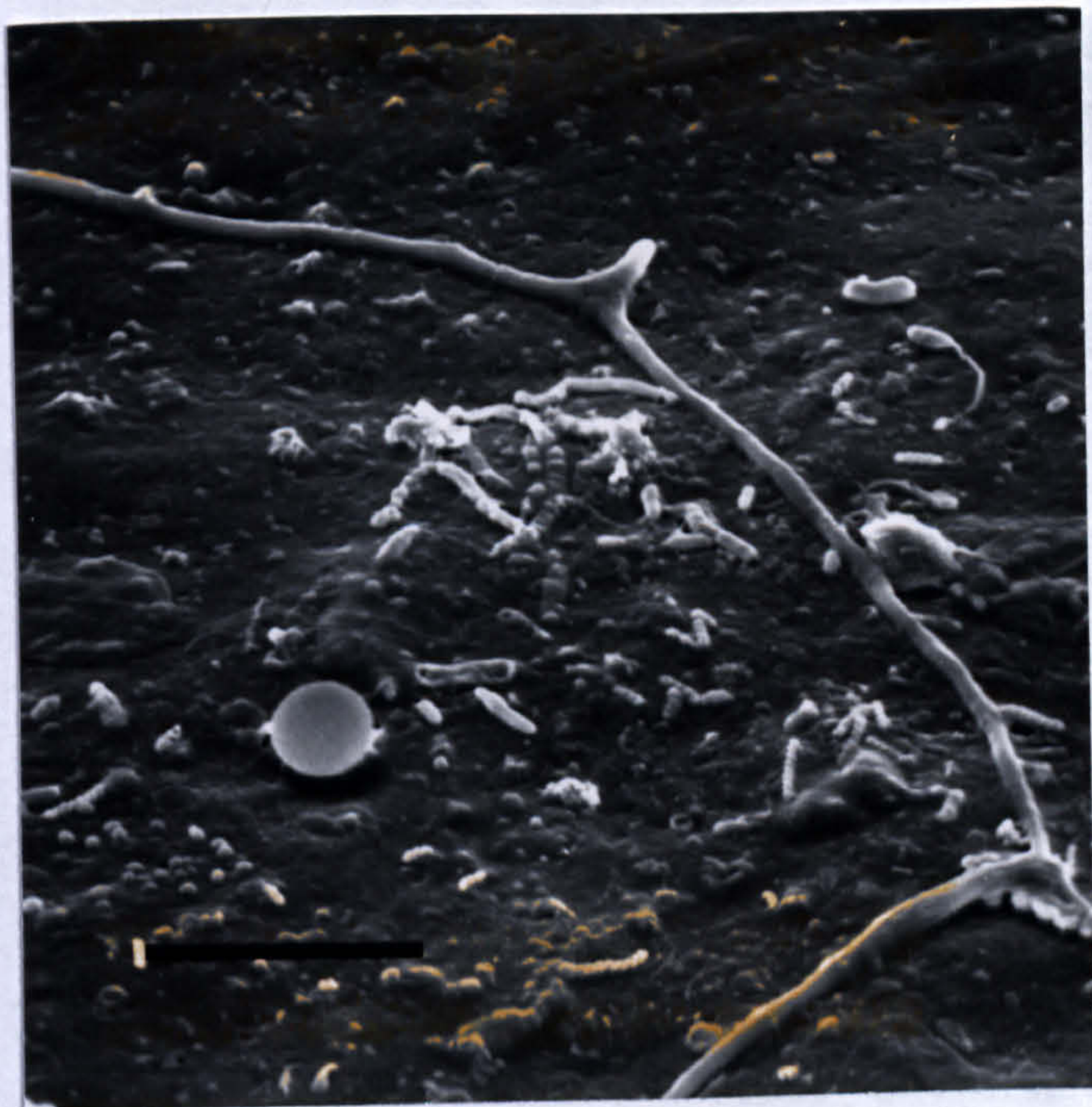
(a)



(b)



(c)



(d)

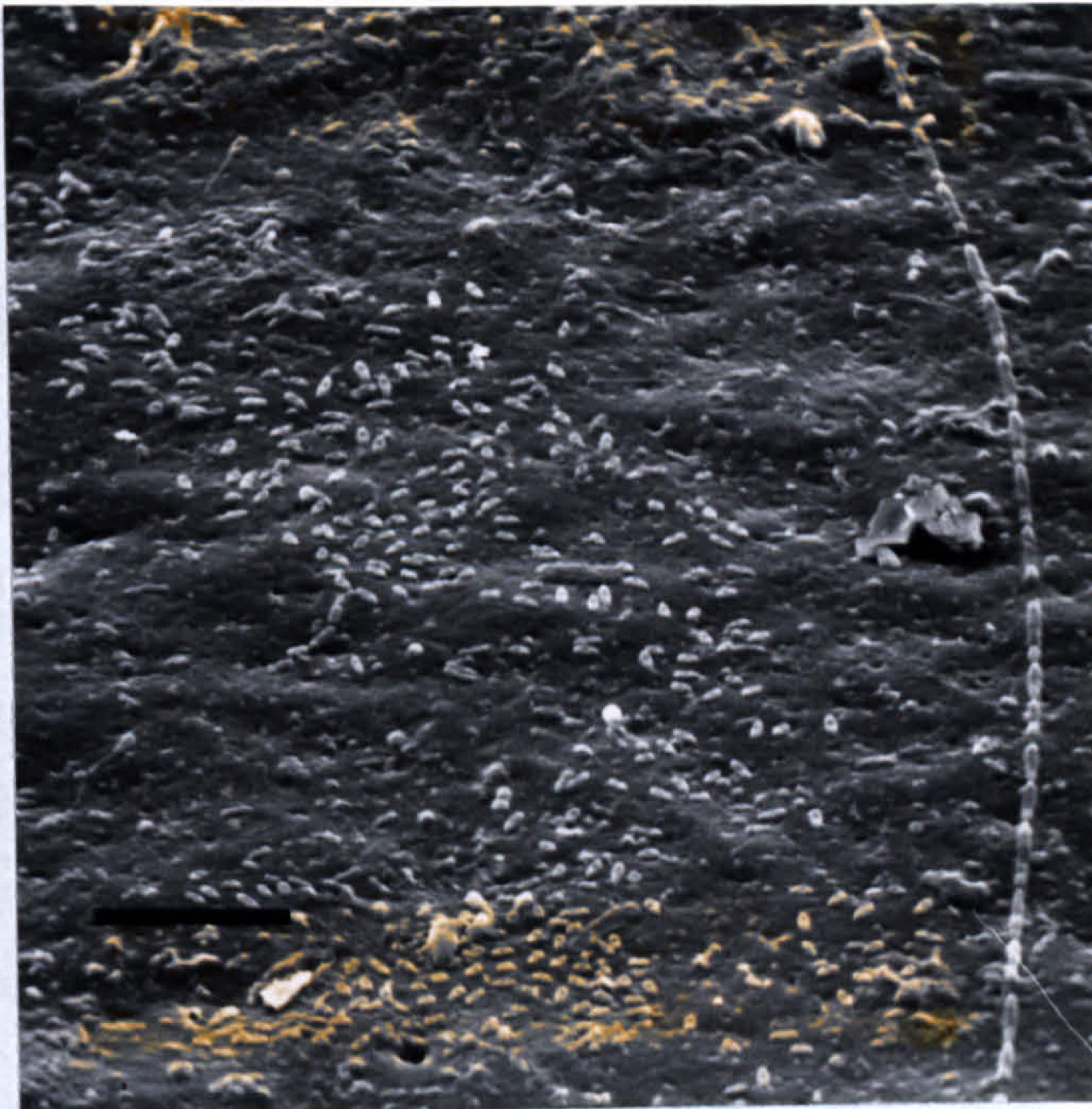
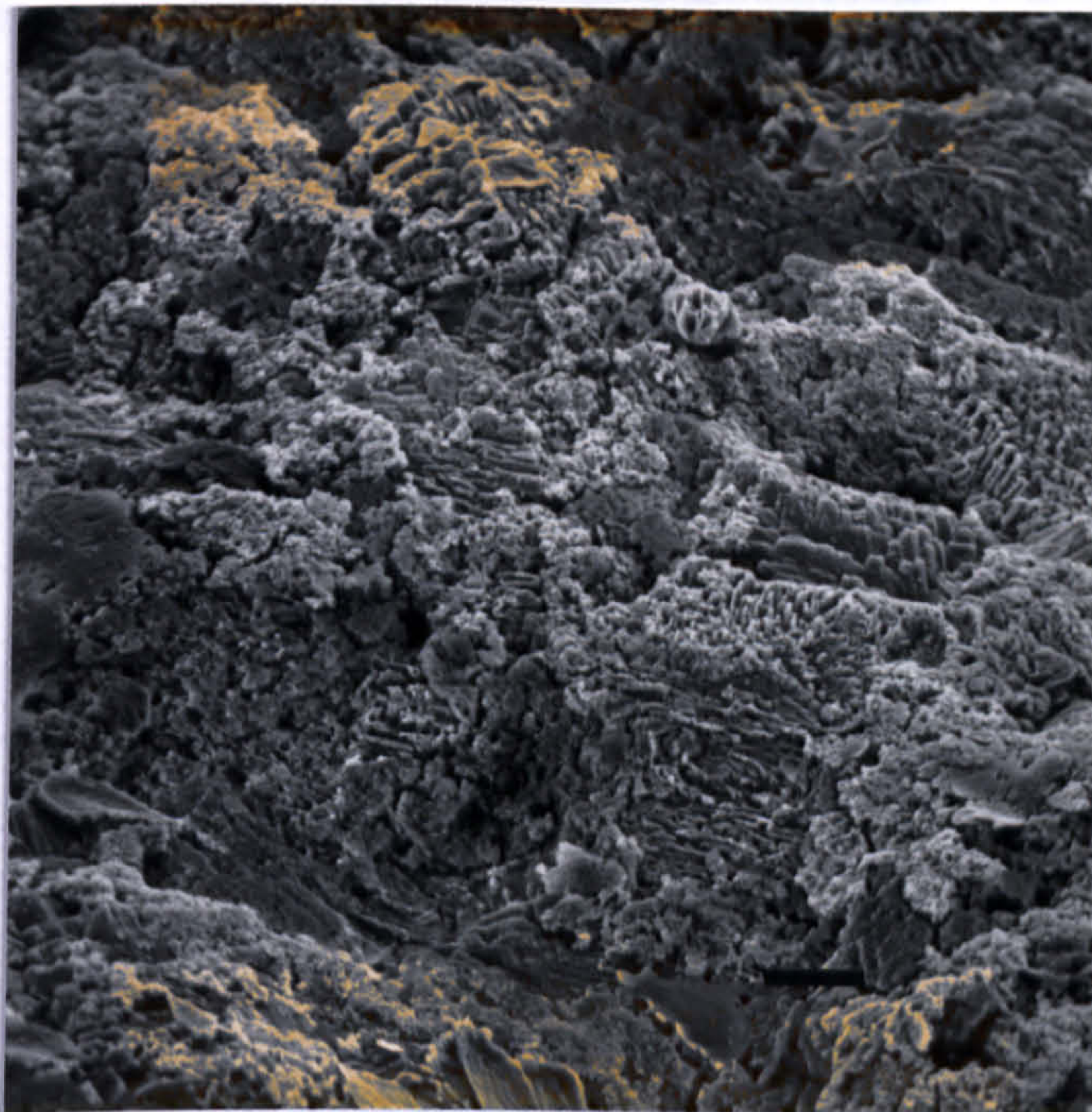
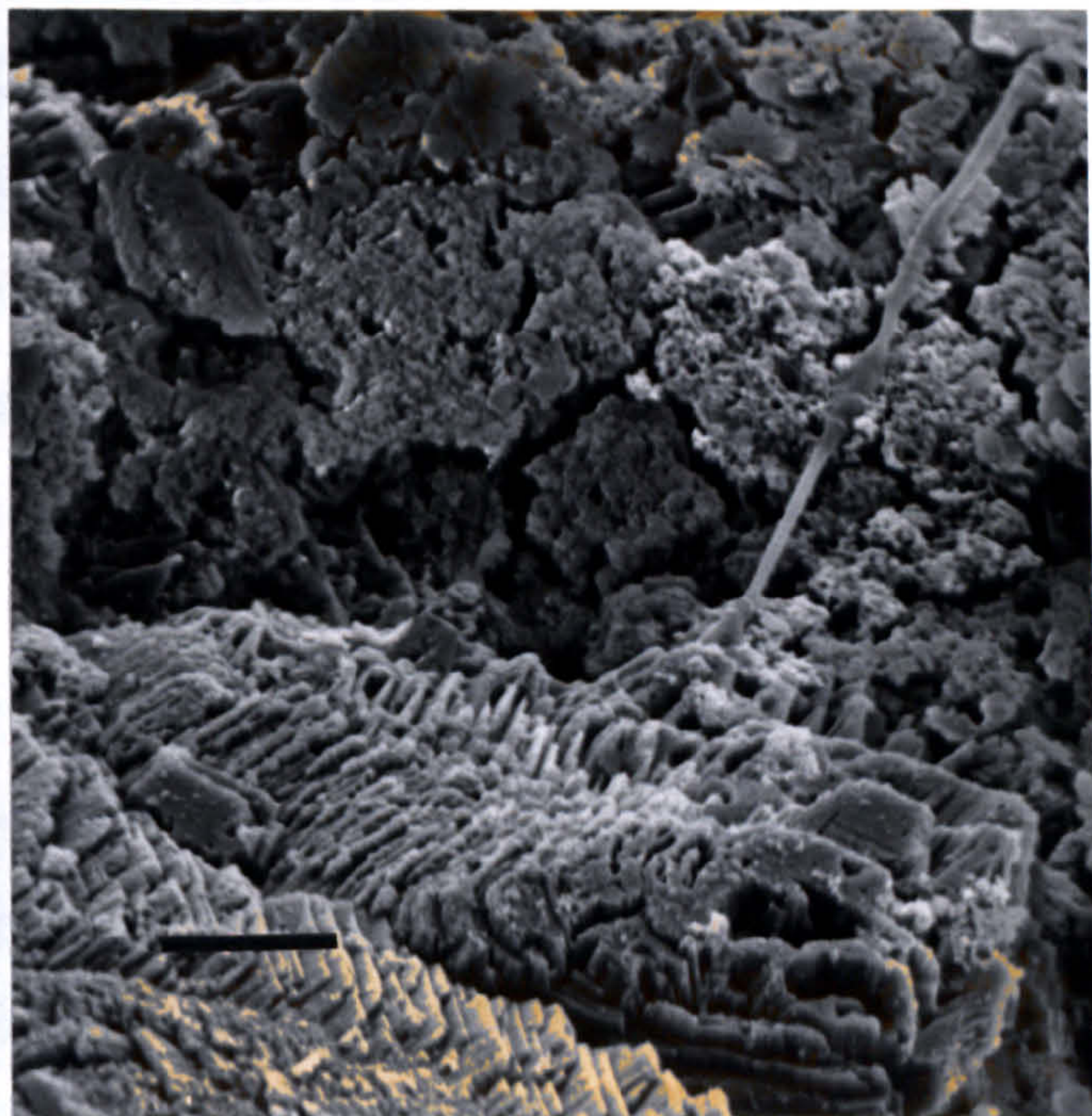


Figure 4.23 Scanning electron micrographs showing the colonisation of mild steel (a and b) and stainless steel (c and d) after 7 days at 30°C. Bar denotes 10µm.

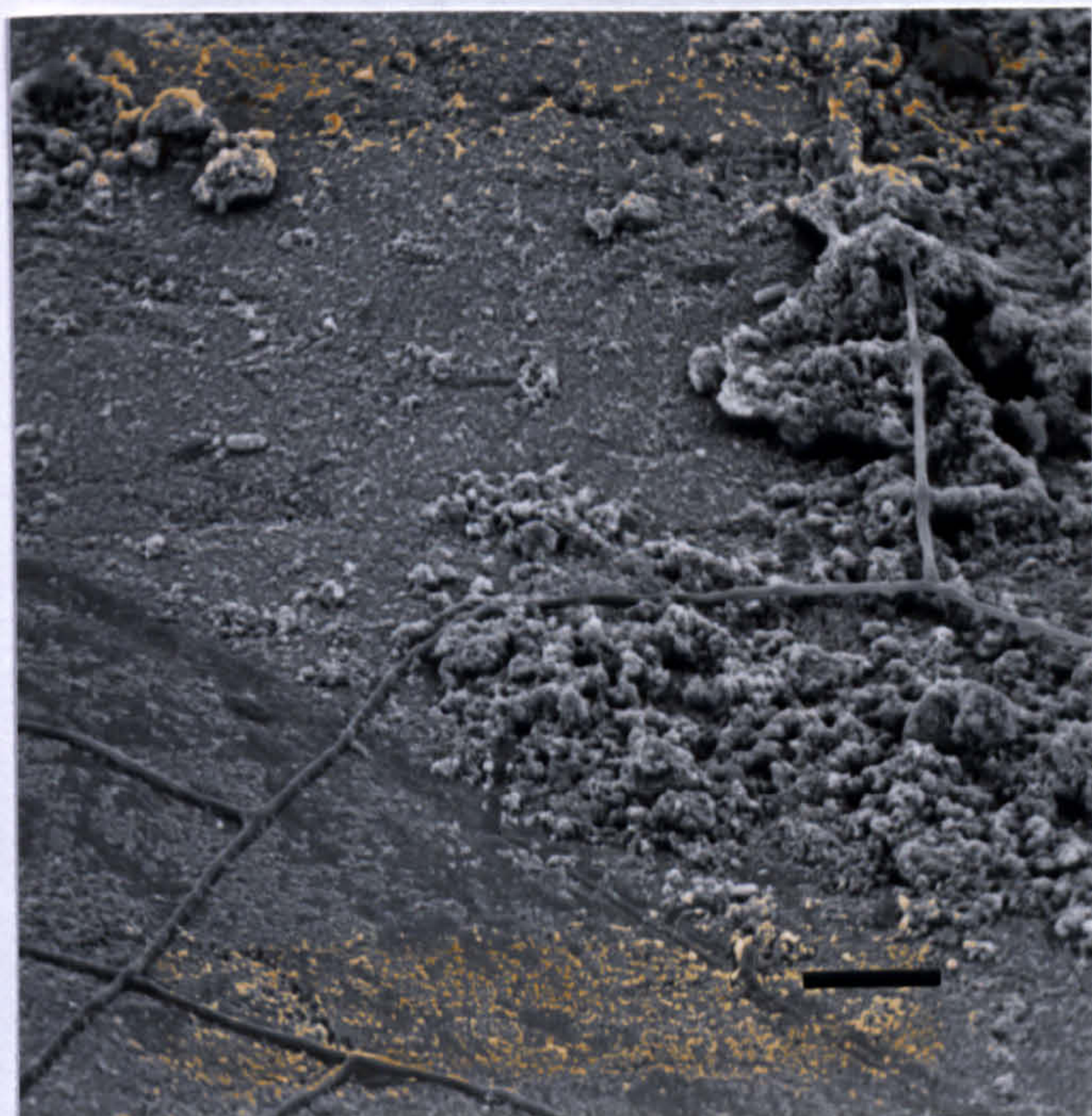
(a)



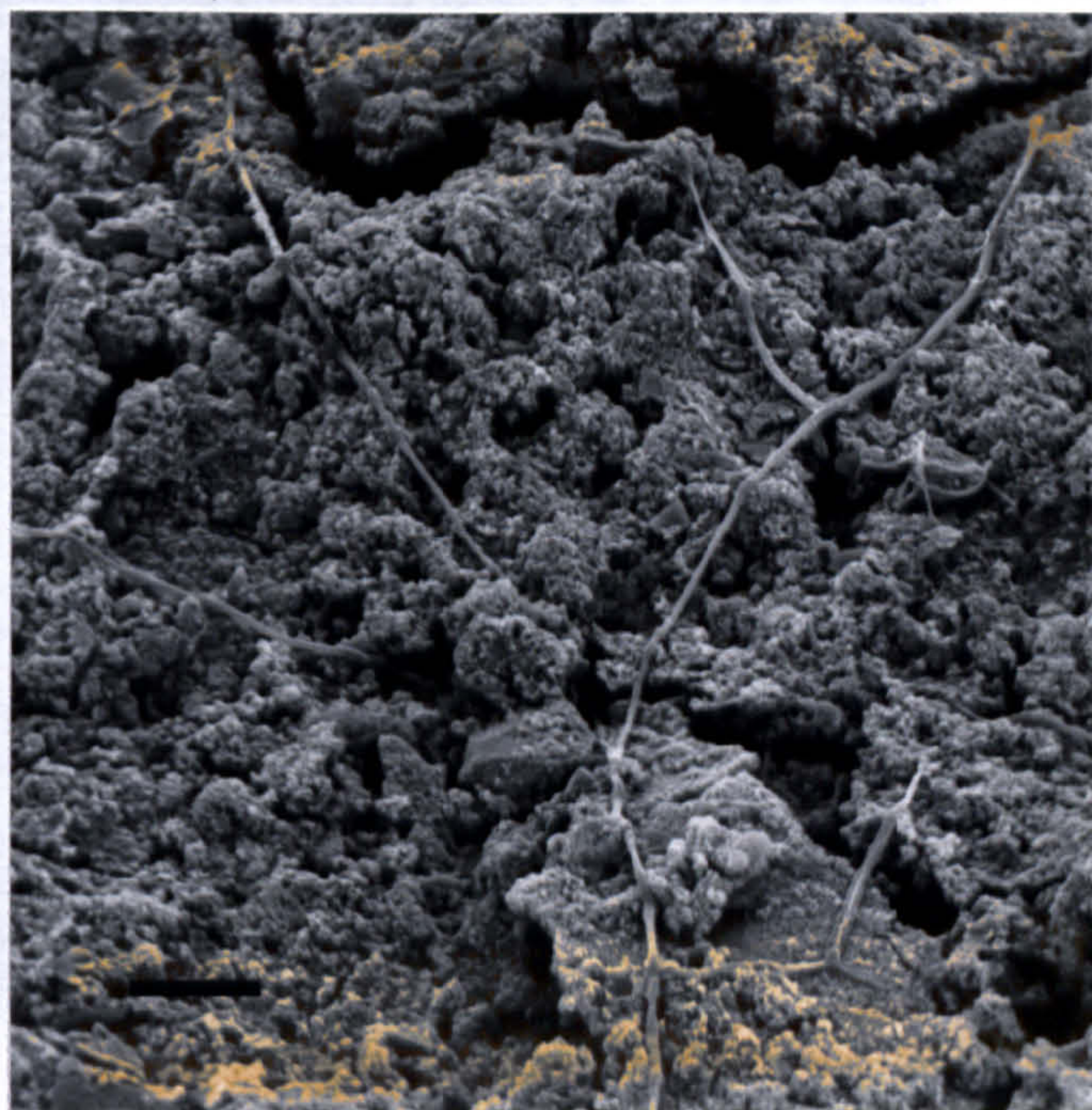
(b)



(c)



(d)



4.3.7 Comparison of the colonisation of the test materials

The results indicate that materials support the growth of microorganisms to varying degrees, dependent on the nature of the substrata. When mean values were compared, all of the plumbing materials were found to have higher total colonisation and numbers of *L. pneumophila* than glass (immersed into the model system with no other materials) (Table 4.2).

The elastomeric materials supported highest numbers of total flora and *L. pneumophila* but the increase in the total biofouling was greater than the increase in numbers of *L. pneumophila*. Plastics supported increases in biofilm flora from 2.4-14.5 times that occurring on the glass surface. The numbers of *L. pneumophila* in the biofilms on the plastic surfaces were not directly related to the increases in total flora, for example, PVCc had a 3-fold increase in total flora compared with glass but a disproportionate increase in the *L. pneumophila*, which was 13 times greater. Stainless steel was the least colonised of the plumbing materials but increased the numbers of *L. pneumophila* within the biofilm above those of PVCu and polyethylene despite the fact they supported a greater total flora.

4.3.8 Comparison of the numbers of microorganisms in the biofilm and planktonic phases of the model system

The mean numbers of microorganisms present in the planktonic and the biofilm phases were compared (table 4.1). In most cases the biofilm was found to contain greater numbers per unit area than the planktonic phase per unit volume. Total viable flora appeared to concentrate on the surfaces of PVCu, mild steel, ethylene-propylene, and latex. The *L. pneumophila* occurred as a higher proportion in the biofilms on polypropylene, PVCc, PVCu, ethylene-propylene and latex.

4.3.9 The extent of leaching of nutrient from the plastic and elastomeric materials

Table 4.5 indicated that numbers of microorganisms in the planktonic phase were elevated in the presence of plastic or elastomeric materials. This implied that the leaching of nutrient from the materials could possibly lead to enhanced growth in the planktonic phase. The possibility was investigated and Table 4.6 shows that all of the materials, with the exception of glass resulted in elevated TOC. The latex, ethylene-propylene and polyethylene surfaces increased the concentration of organic carbon in excess of 150 ppm. Copper and the other plastic surfaces only slightly increased the amount of TOC in the water.

Table 4.5 Extent of leaching of the various plumbing materials determined by increased biofilm formation on the glass control surfaces immersed in the presence of the test materials.

Glass in the presence of	Mean colonisation (cfu cm ⁻²)		Colonisation ratio (cfu cm ⁻²)	
	of flora ^a	of <i>L.pneumophila</i> ^a	for total flora	for <i>L.pneumophila</i>
Untreated	7.00x10 ⁴	1.00x10 ³	1	1
Latex	1.14x10 ⁶	3.70x10 ³	16.3	7.8
Polypropylene	3.14x10 ⁵	7.8x10 ³	4.5	7.8
Ethylene-propylene	2.98x10 ⁵	2.00x10 ²	4.3	0.2
PVCu	3.48x10 ⁵	2.93x10 ³	5	2.9
Polyethylene	4.60x10 ⁵	8.00x10 ²	6.7	0.8
PVCc	2.35x10 ⁵	1.9x10 ³	3.4	1.9
Stainless steel	not done			
Mild steel	not done			

The colonisation ratio is the cfu/cm² of the total microbial flora or legionella recovered from glass immersed in the presence of each material and referenced to glass alone.

Table 4.6 Total carbon leached from materials exposed to water for 3 days.

Material in water	TOC(ppm)
Glass control	2.78±0.4
Copper	4.15±0.17
Polybutylene	4.46±0.15
PVCc	6.02±0.11
PVCu	5.42±0.11
Polypropylene	5.98±1.56
Polyethylene	179±0.82
Ethylene-propylene	157±0.84
Latex	320±19.4
Stainless steel	not done
Mild steel	not done

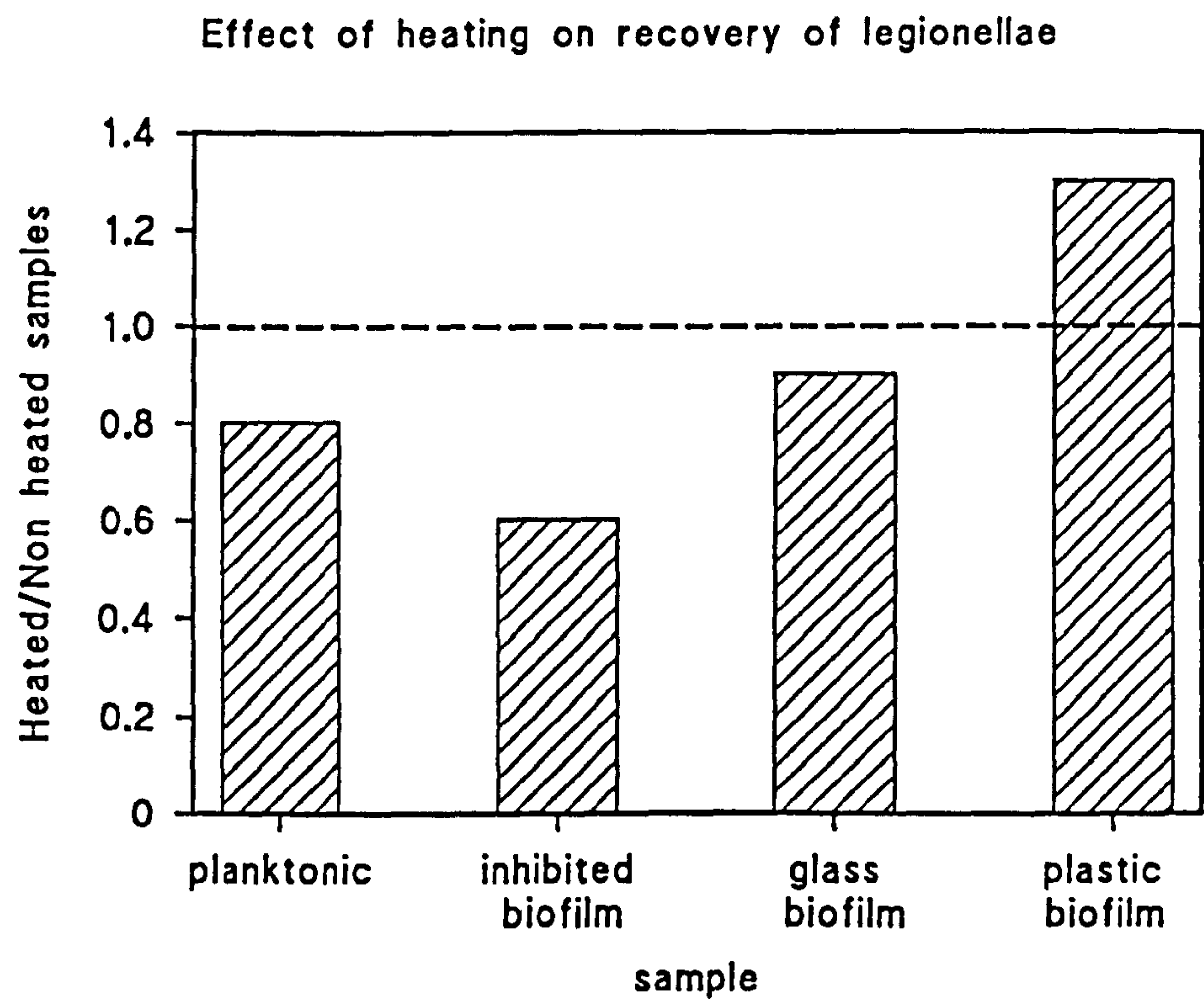
values give a mean of three determinations ± SD

4.3.10 Recovery of *L. pneumophila* from different materials

In recovery of *L. pneumophila* from planktonic and biofilm samples, those samples heated for 15 mins were found to contain lower numbers of the pathogen than unheated samples. Therefore, heat treatment was modified to only 10 min at 50°C. The heating of the samples of the biofilm and planktonic phases prior to plating onto GVPC resulted in a reduction in background flora. In some cases legionellae could then be recovered from samples which could not have been found in unheated samples due to the confluent growth of other flora. Where the counting of legionellae colonies was possible for both heated and non heated samples a ratio of recovery was calculated. The samples were divided into four groups, samples from the chemostat culture (planktonic), biofilm samples from surfaces known to be inhibitory to growth ie. copper and silver paint (inhibitory), biofilm samples from glass surfaces (glass) and biofilms from plastic surfaces (plastic). The ratio was calculated by the sum of all legionellae in heated samples in the group, divided by the sum of all legionellae in non heated samples in the group.

The results are shown in Figure 4.24. Biofilms on glass surfaces and planktonic samples showed a slight overall reduction in recovery (0.9 and 0.8 respectively). Plastic surfaces supported thicker biofilms containing higher numbers of *L. pneumophila*. The recovery of legionellae from these samples improved on heating to 1.3 possibly due to dispersal of microcolonies of *L. pneumophila*. The inhibitory surfaces supported sparse biofilms and recovery was reduced following heating to 0.6 compared to the unheated samples. The combined metal toxicity and heat stress possibly reduced recoverable legionellae.

Figure 4.24 The effect of heating on the recovery of *L. pneumophila* from biofilm and planktonic samples.



4.4 DISCUSSION

The materials examined in this investigation were all found to support biofilms and all of these biofilms contained *L. pneumophila*. Each of the materials were colonised by differing pioneering species despite the presence of the same bacterial population in the aqueous phase. The biofilms that developed on the different surfaces varied in diversity, abundance and morphology. Materials colonised under the same environmental conditions are therefore influenced by the supporting material. The biofilm serves as a focal point where bacteria and protozoa interact. The pioneering population will modify the surface conditions to enable bacterial succession to take place. The resultant biofilm may aid the colonisation of the surface by *L. pneumophila* by supporting bacterial flora that provide nutrient or by encouraging protozoal populations which can act as hosts for the pathogen.

Colonisation of steel surfaces

The stainless and mild steel surfaces supported high numbers of legionella and this was attributed to the supply of iron to the chemostat model resulting in enhanced growth of the pathogen. The rusting of the mild steel surface occurred rapidly and the possibility of microbially induced corrosion was considered as one reason for the rapid corrosion. However, sterile mild steel coupons suspended in sterile water from the model system were found to rust as rapidly at 30 °C indicating the microbial population was unlikely to have a significant role in the deterioration of the metal tiles under these conditions.

The numbers of *L. pneumophila* on the surface of mild steel coupons was greater than that on the stainless steel. This could possibly be attributed to increased availability of iron due to the corroding of the mild steel. Iron is an essential growth nutrient and is limited availability in aquatic systems (Reeves *et al.*, 1981).

Colonisation of elastomeric surfaces

The elastomeric surface had considerable growth of micro-organisms on the surface and this correlated with increased total carbon in the leaching experiments. Latex material surfaces supported 289 times more total flora than glass surfaces. The legionella did not occur within the latex biofilm as a high proportion of the total flora, their numbers increased in the biofilm to nearly 130 times those occurring in the glass biofilm. The material did not support the same increase in the numbers of legionella as it did the total flora. This is most likely due to the population present within the biofilm inhibiting the numbers of legionella in some way, either not supplying sufficient nutrient or by producing inhibitory substances. The results were to be expected since natural latex has been withdrawn from use in water systems due to the growth of bacteria on the surface and the surface was deliberately included as a positive control.

The ethylene-propylene formulation tested supported a maximum flora of 2.9×10^7 after 28 days, and was included as an example of a washer material. The numbers of legionella in the biofilm had a disproportionately high increase compared with the increase in total flora when compared with glass.

The photomicrographs showed that biofilms on both elastomeric surfaces were cracked. The drying process necessary for preparation for observation under the scanning electron microscope had resulted in the reduction of biofilm thickness and cracking may have been an artifact. It had been noted, however, that as the materials had been immersed into the sterile water to remove unwanted planktonic bacteria, aggregates of biofilm were lost to the washing liquid. These particles had a size of approximately 1mm diameter and would have contained high numbers of microorganisms that were excluded from plate counts. The loss of these particles would indicate that removal of biofilm from the water and exposure to air may result in sufficient dessication to allow

sloughing of biofilm when reimmersed into the aquatic environment.

Colonisation of plastic materials

The plastic surfaces were intermediate in the total colonisation between the copper and the materials elastomeric surfaces. The plastics exhibited a range of carbon leaching from their surfaces and this may have been a contributory factor in their increased colonisation when compared with the control glass surface.

Polypropylene and PVCc materials supported biofilms with PVCc having the most abundant of all of the plastic tubing materials tested. The total flora reached a maximum of 1.7×10^6 c.f.u cm⁻² after only one day, this was at least in part due to the leaching of nutrient from the surface. The maximum number of legionella in the cPVC biofilm was higher than would have been predicted from the increase in the numbers of total flora. The maximum reached after 7 days was 7.85×10^4 c.f.u cm⁻². The increase in the proportion of the legionella in the biofilm may have been due to them being encouraged by the growth of other biofilm bacteria. The amoebae present on the surfaces of this material may also have increased the numbers of legionella by acting as hosts for bacterial growth, their presence on this surface is probably due to the presence of bacterial species favoured as food sources by the protozoa (amoebae and ciliates). The polypropylene supported only 2.4 times more colonisation than the glass control but *L. pneumophila* numbers were 12 times greater.

The polyethylene supported a disproportionate increase in total flora compared with numbers of *L. pneumophila*. The polyethylene surface supported 14.5 times more total flora than the glass control surfaces and the legionella were 4 times greater. The PVCu surfaces had increases in the total flora that were proportional to the increases in the numbers of legionella in the biofilms. The numbers of *L. pneumophila* on the PVCu were 5 times greater than that occurring on glass surfaces.

Factors influencing biofilm formation

There are several reasons for the preferential attachment of micro-organisms to one surface compared with another. The experiments only considered the increased nutrient availability due to leaching of TOC from the test material. One of the other important factors may include the protection of the micro-organisms from shear. Some of the materials had crevassed surfaces which may have aided colonisation by pioneer species of microorganisms. Crevices in the polypropylene surface were observed to be rapidly colonised and biofilm build up from these small microcolonies. Surface properties including surface charge and hydrophobicity could also influence to colonisation of the materials.

Factors influencing inclusion of *L. pneumophila* into biofilms

The results presented here demonstrate that there is no direct relationship between the total biofilm development and the number of *L. pneumophila* incorporated into the biofilm. The number of legionellae is dependent upon the bacterial species that are incorporated into the biofilm which may either aid the survival and growth of the legionella, or may inhibit them.

The bacteria present will also determine the number and type of protozoa present on the biofilm. Protozoa are selective feeders that naturally occur in the biofilm rather than in the planktonic phase and the effect of them on the biofilm is complex. They may reduce the extent of biofilm development by controlling bacterial numbers by grazing and are also likely to determine species composition of the remaining populations. Some species of amoebae and other protozoa have been shown to support the growth of legionella. Therefore they may increase the proportion of the legionella within the biofilm by not only increasing the numbers of legionella but by also reducing the other flora.

Whether eukaryotes are important or not in the persistence of legionella in water systems, material selection influences biofouling and colonisation by *L. pneumophila*.

Recovery of *L. pneumophila* from biofilms

The pretreatment of samples by heating has been shown to be beneficial in increasing the recovery of legionella in biofilms on the surface of plastic materials. The heat treatment of samples reduced the number of colonies of other aquatic microorganisms and made isolation of *L. pneumophila* easier by reducing competition on solid media. The improvement in the counts may be attributed to the heat dispersing microcolonies of *L. pneumophila* or perhaps the release of microorganisms from within amoebae or cysts. Recovery of *L. pneumophila* from biofilms on glass surfaces was slightly reduced following heat treatment and this was thought to be due to heat stress. The decimal reduction time of *L. pneumophila* has been shown to be 111 min at 50 °C by Dennis *et al.* (1984) and although the organism shows greater stability than other flora, it appears to be adversely effected by the elevation of temperature. A similar reduction in recovery of the organisms was observed for the planktonic samples.

When biofilms from the surface of materials known to be inhibitory (copper and silver paint) were tested a more dramatic reduction was observed. Low recovery was possibly due to combined stress of growth under adverse conditions and heat resulting in a synergistic effect.

The data would suggest that the methods of plating of heated and non heated samples used for planktonic specimens is also required for the detection of legionella in biofilm samples. However, a reduction in the incubation period to 10 min at 50 °C results in a greater number of colonies of *L. pneumophila*, if quantification is required rather than detection alone.

Biofilm and planktonic interactions

Water systems are composed of two distinct but interactive phases, the aqueous phase and the biofilm present on the pipe surfaces. The data shows that there is no direct relationship between numbers of biofilm microorganisms and numbers of microorganisms in the planktonic phase. The interactions that occur between the biofilm and the planktonic phase are influenced by the material on which the biofilm forms. Fluctuations that occurred in the populations of micro-organisms within the biofilm over the experiments correspond to changes in the planktonic bacteria.

Biofilms are inevitably present on the surface of water systems so control of potential pathogens may be best achieved, if those microorganisms present, formed biofilms which were not easily sloughed, where the biofilm flora was inactive and the biofilm structure was stable.

Sloughing of the biofilm is particularly undesirable as the loss of clumps of biofilm into the planktonic phase may result in enhanced colonisation of the whole system by *L. pneumophila*. The occurrence of *L. pneumophila* in microcolonies within the biofilm with the microorganisms it requires for growth, ensures two major benefits for the survival and growth of the pathogen. The first benefit is that the pathogen is protected by the other members of the community from biocide treatment in the aqueous phase and therefore giving a great chance of recolonisation further down the pipe system. *Legionella pneumophila* receives a competitive advantage by this type of community migration since they are accompanied by a bacterial consortium capable of supporting their growth enabling rapid colonisation of the new location.

There are two important consequences of sloughing of cells, one is migration and the other is the preservation of water (and nutrient) channels in the biofilm structure. If bacteria accumulated on the surface of the material without cells migrating, the biofilm

would form a dense mat where conditions would be unfavourable for many species. Nutrient channels are essential if a climax community is to be composed of aerobes. Two forms of migration are likely, one occurs passively with the random sloughing of portions of biofilm. The second is stimulated when accumulation of toxic products or lack of diffusion of nutrients cause hostile conditions for the bacteria which stimulate active migration of cells to more favourable conditions. The processes that occur within the biofilm are dynamic and the loss of biofilm by sloughing exposes areas of the substratum surface and thus making that area available for colonisation at a later time.

Material selection is likely to influence the dynamic interactions that occur between the planktonic and biofilm phases, by influencing the community formed. Could it be a possibility that those materials which show the greatest initial colonisation are therefore the most likely to accumulate thickest biofilm, this in turn will slough at the greatest rate. If this were true, then it may be that those which formed sparse, stable biofilms would be least likely to continually release *L. pneumophila* for recolonisation or infection.

CHAPTER 5

THE INFLUENCE OF WATER TEMPERATURE ON THE GROWTH OF *LEGIONELLA PNEUMOPHILA* AND ON BIOFILM DEVELOPMENT

5.1 INTRODUCTION

The widespread occurrence of *L. pneumophila* in natural and man-made environments has given a good indication of the ability of the organism to grow and survive over a wide temperature range. In natural environments *L. pneumophila* was detected by immunofluorescence between temperatures of 5.7 to 63 °C during an extensive survey by Fliermans *et al.* (1981).

In man-made ecosystems the occurrence of the pathogen appeared to be linked with hot water systems. A survey of water systems was completed by Ruf *et al.* (1988) following an outbreak of nosocomial infections. Of 171 cold water samples, with a temperature range of 14 to 27 °C, 43.2 % were found to contain *L. pneumophila*. In contrast, 242 of 282 hot water samples were demonstrated to contain *L. pneumophila*. The hot water temperature ranged from 35 to 69 °C.

Yee and Wadowsky (1982) used two, naturally occurring, mixed populations to examine the effect of temperature on growth at 5, 25, 37, 42, and 45 °C. Optimum growth occurred at 37 °C but growth was also achieved at 42 °C. No growth occurred at 45 °C. Additional work using a similar mixed consortium was achieved by the serial transfer of inocula in tap water (Wadowsky *et al.*, 1985). Growth was shown to occur at 25, 32, and 37 °C but in these experiments death of the organism occurred at 42 and 45 °C. Further evidence of the ability of the *L. pneumophila* to grow at elevated temperatures was then provided by Tison *et al.* (1980). These authors were able to demonstrate that in coculture with the cyanobacterium *Fischerella* sp., *L. pneumophila* was able to grow at 45 °C.

Dennis *et al.* (1984) tested the decimal reduction time (the time required to kill 90% of the organisms) of several species of legionella and other environmental isolates at 46,

50, 54, and 58 °C. At 46 °C the legionella numbers were not significantly reduced. For *L. pneumophila* a decimal reduction time for 50 °C (D_{50}) was 111 min, a D_{54} of 27 min and a D_{58} of 6 min. Most of the environmental isolates survived less well at elevated temperatures including a pseudomonad, a micrococcus and a coliform. However, a strain of *Sarcina* was found to be more tolerant of high temperature. The decimal reduction time for 60 °C was calculated to be 1.3 to 10.6 min for pure cultures of *L. pneumophila* by Stout *et al.* (1986).

Although the organism had been shown to be able to grow and survive in a broader range of temperatures than many aquatic microorganism, the evidence suggested that there was a maximum temperature for growth and this was the principal used to control the organism in man made water systems. The DHSS Code of Practice issues guidelines for the operations of hot water systems to ensure that systems are not colonised. Hot water should be stored at 60 °C and the returning water should be maintained above 50 °C. The water from any tap in the circuit should reach 50 °C within 1 min of flushing. Cold water systems should be maintained below 20 °C in order to discourage excessive growth within the systems. Most often cases of infection have appeared to occur when the operating temperature of hot water systems is below that of the suggested guidelines. In a study of the cause of 13 fatal cases of nosocomial *L. pneumophila* Ruggenini *et al.* (1989) attributed infection to the growth of the *L. pneumophila* in the hot water system which operated at 35 °C. Maintenance of high operating temperatures has been largely successful in the elimination of *L. pneumophila* from hot water systems. For example, by heat treatment of 60 - 77°C for 72 h followed by maintenance of temperatures of 54 °C Best *et al.* (1983) were able to eliminate the problems of colonisation. However, the organism can be persistent despite temperature treatment. Colbourne *et al.* (1984) were unsuccessful in removing *L. pneumophila* from a hospital by operating the system at 55 °C and this was due to survival of the organism within biofilms on washers. Another incident where the elevation of temperature failed to eliminate *L. pneumophila* from a hospital hot water system was

investigated by Groothuis *et al.* (1985). The failure of the heat treatment was attributed to the presence of dead ends of pipe work which were not reached by the hot water used for decontamination. The *L. pneumophila* present in these dead ends was then responsible for the recolonisation of the remaining system when operating temperatures were reduced.

All of the work so far carried out on the temperature tolerance of *L. pneumophila* has considered only the planktonic phase. The suggestion of Colbourne *et al.* (1984) that biofilm could enhance the potential for survival and the failure of heating to be successful in some cases would indicate that a study of the effect of temperature on biofilm formation may be of interest. The model system that has been used for the influence of material selection is adaptable for the examination of temperature effects. The first vessel ensures a constant supply of inoculum to the second, biofilm generating vessel, where temperature conditions can be modified. Since the material selection has been shown to have an important role in the growth of *L. pneumophila* the effect of temperature and material was simultaneously investigated. Temperatures examined were 20, 40 50 and 60 °C. The growth at 30 °C had already been examined and it was felt the most suitable temperatures to study were those that occur in water systems. A cold water system may reach 20 °C in summer, 40 to 60 °C covers the range of temperatures of operational hot water systems despite the guidelines. The problem of maintaining the water systems at the optimum for eradication of *L. pneumophila* is in conflict with the desire to reduce energy cost and the advantages of lower water temperature include the prevention of patient scalding (Plouffe *et al.*, 1983). The lowering of water temperatures from 60 °C to 40 to 45 °C to economise on energy consumption resulted in colonisation of the water systems by *L. pneumophila*.

The overall aims of this section of the investigation are to determine what influence temperature had on both biofilm development and growth of *L. pneumophila*. Another aim was to determine if the effect of material selection had any influence on the impact

of the temperature of biofilm formation. To these ends three materials were selected, copper (to determine if inhibition could be sustained over a wide temperature range) and two commonly used plastic materials, polybutylene and cPVC.

5.2 MATERIALS AND METHODS

5.2.1 The effect of temperature on biofilm and planktonic growth

The chemostat model described in Section 2.1 was used to investigate the influence of temperature on biofilm growth on copper, cPVC and polybutylene surfaces. The chemostat was operated using a hard yorkshire water as the sole nutrient source. The chemostat model was operated as described in Section 2.1.3 with the exception that the stirrer speed was maintained at 300 rpm since the large variation in the temperature range resulted in large variations in stirrer speeds in order to maintain 20 % DOT and this may have altered biofilm formation.

The colonisation on polybutylene was examined at 20, 40, 50 and 60 °C. The chemostat temperature was gradually increased by 5 °C a day until the desired temperature was obtained. Planktonic samples were removed from the biofilm generation vessel at daily intervals until the numbers of total flora and *L. pneumophila* appeared to have stabilised. In all cases, the vessel was allowed to recover from temperature modification for a minimum of two weeks. Once the chemostat culture was sufficiently adapted to the new operating temperature the tiles of polybutylene were inserted into the chemostat culture as described in 2.2.1. These were removed from the vessel after biofilm had been allowed to develop on the surfaces for 1, 4, 7, 14, and 21 days. The planktonic and biofilm samples were assessed microbiologically as described in 2.3. The biofilms were also examined using the scanning electron micrograph as described in 2.7.1.

The population profiles of the microorganisms in the biofilms developed at the various temperatures were evaluated as described in 2.4.

The experiment was repeated using PVCc and copper as materials to support biofilm development. The only modification to the protocol was that the colonisation was

evaluated for PVCc at 60, 50, 40 and then 30 °C and copper at 30, 40, 50 and then 60°C.. The chemostat culture was allowed to recover from changes in operating temperature as described for polybutylene.

5.2.2. Populations in biofilms at different temperatures

The microorganisms present in the biofilms on the various material surfaces were counted and identified as described in section 2.4 so that population profiles could be constructed. The organisms present within the biofilms were then divided in to legionella, pseudomonads and other gram negative organisms (OGN) so that the diversity of organisms could be evaluated.

5.2.3 Polymer production at different temperatures

Glass surfaces were inserted with the materials so that polymer production at different temperatures could be evaluated. The glass tile supporting the biofilm was air dried and then treated using the combined alcian blue PAS staining method described in Section 2.6.4.

5.3 RESULTS

5.3.1 Growth in the planktonic phase.

At 20°C the planktonic phase contained 1.3×10^4 to 7.56×10^5 cfu ml⁻¹ of non-legionella and this was least when copper was present in the model system (Table 5.1). The presence of copper also reduced the numbers of *L. pneumophila* to below the limit of detection of 10 cfu ml⁻¹ (Table 5.2). When plastic materials were present *L. pneumophila* accounted for a low proportion of the planktonic flora, approximately 0.1%.

The planktonic populations of non-legionella varied between 1.42×10^4 to 3.90×10^5 cfu ml⁻¹ at 40°C. *Legionella pneumophila* occurred in the planktonic phase at a concentration of 2.99×10^2 and 7.50×10^3 cfu ml⁻¹ and accounted for up to 12% of the population. The presence of copper in the aqueous phase reduced both the total population and the numbers of *L. pneumophila*.

At 50°C the total non-legionella population was between 2.00×10^4 to 3.35×10^6 cfu ml⁻¹. *Legionella pneumophila* was less than 0.7% of the total population, being between 1.00×10^1 to 3.45×10^2 cfu ml⁻¹. Copper was again observed to reduce both the total planktonic population and the numbers of *L. pneumophila*.

Legionella pneumophila was absent from the planktonic phase at 60°C. The numbers of planktonic phase bacteria was low at this temperature, being between 8.45×10^2 to 8.39×10^4 cfu ml⁻¹, with lowest numbers occurring when copper was present.

Table 5.1 Comparison of total non-legionella in biofilms forming on different plumbing materials, on glass control surfaces and in the planktonic phase at different temperatures.

Experiment	Mean total number of non-legionellae		
	on material surface	on glass control surface	in planktonic phase
	(cfu/cm ²)	(cfu/cm ²)	(cfu/ml ⁻¹)
20°C			
Copper	2.16x10 ⁵	3.08x10 ⁵	3.79x10 ⁴
Polybutylene	5.70x10 ⁵	6.23x10 ⁵	2.87x10 ⁵
PVCc	1.81x10 ⁶	5.05x10 ⁵	2.63x10 ⁵
40°C			
copper	8.04x10 ⁴	6.66x10 ⁴	9.18x10 ⁴
Polybutylene	1.18x10 ⁶	4.43x10 ⁵	4.30x10 ⁴
PVCc	3.67x10 ⁵	1.40x10 ⁵	3.68x10 ⁵
50°C			
copper	2.26x10 ⁴	1.20x10 ⁵	2.40x10 ⁴
Polybutylene	3.21x10 ⁶	1.5x10 ⁶	8.43x10 ⁴
PVCc	1.22x10 ⁵	3.78x10 ⁵	6.43x10 ⁴
60°C			
Copper	4.47x10 ²	6.29x10 ²	1.83x10 ³
Polybutylene	4.25x10 ⁴	1.25x10 ⁴	5.84x10 ³
PVCc	5.19x10 ³	1.78x10 ⁴	6.06x10 ⁴

Means were calculated from all values determined over 1-21 days

Table 5.2 Comparison of numbers of *L. pneumophila* into biofilms on different plumbing materials, on glass control surfaces and in the planktonic phase.

Experiment	Mean number of <i>L. pneumophila</i>		
	on material surface (cfu/cm ²)	on glass control surface (cfu/cm ²)	in planktonic phase (cfu/ml ⁻¹)
20°C			
Copper	BD	BD	3.00x10 ⁰
Polybutylene	6.65x10 ²	2.79x10 ²	1.28x10 ²
PVCc	2.13x10 ³	4.69x10 ³	2.81x10 ²
40°C			
copper	1.67x10 ³	7.89x10 ²	8.17x10 ²
Polybutylene	1.12x10 ⁵	1.59x10 ⁴	2.20x10 ³
PVCc	6.84x10 ⁴	4.64x10 ³	2.20x10 ³
50°C			
copper	BD	2.00x10 ⁰	1.70x10 ¹
Polybutylene	8.58x10 ²	3.31x10 ²	1.18x10 ²
PVCc	6.00x10 ¹	4.3x10 ¹	7.00x10 ¹
60°C			
Copper	BD	BD	BD
Polybutylene	BD	BD	BD
PVCc	BD	BD	BD

BD denotes that numbers were below the detection limit of 10 cfu cm⁻² or 10 cfu ml⁻¹. Means were calculated from all values determined over 1-21 days.

5.3.2 Growth in biofilms at 20°C.

PVCc: Biofilms most rapidly formed on PVCc, with a total non-legionella population of 1.16×10^6 cfu cm⁻² after only 24h (Figure 5.1). The PVCc sustained the most abundant biofilm flora for the duration of the 21 days, with a maximum of 5.90×10^6 cfu cm⁻². *Legionella pneumophila* were most rapidly incorporated into the biofilm on PVCc (4.11×10^2 cfu cm⁻² after 24 h (Figure 5.2)) and reached a maximum of 6.60×10^3 cfu cm⁻² of *L. pneumophila* after 7 days, accounting for 0.3 % of the total population. The populations of microorganisms on PVCc were similar in diversity to those occurring on polybutylene at all temperatures examined, therefore only data for polybutylene (Table 5.5) is presented in this study as a comparison for copper (Table 5.4).

Polybutylene: The polybutylene surface supported less total bacteria, with a maximum of 9.50×10^5 cfu cm⁻², but this was not significantly lower than that on the PVCc. The biofilm supported 3.50×10^2 cfu cm⁻² *L. pneumophila* after 24h and had a maximum of 2.20×10^3 cfu cm⁻² after 4 days, accounting for 0.11% of the total biofilm flora. The biofilm that developed on polybutylene contained a diverse mixture of Gram negative bacteria, actinomycetes, fungi and protozoa including amoebae. Pseudomonads were the principal pioneering microorganisms comprising 72% of the total bacterial flora (Table 5.5), the most abundant being *P. testosteroni* and *P. paucimobilis* with *P. maltophilia*, but *P. mendocina* and *P. stutzeri* also being present. Actinomycetes accounted for 24% of the total biofilm flora and *Acinetobacter* sp. occurred in low numbers. The biofilm flora was increasingly dominated by the pseudomonads with 96% of the climax community being attributed to seven species of pseudomonad with increased diversity due to the addition of *P. aeruginosa*, *P. fluorescens* and *P. vesicularis*.

Copper: Copper was consistently less colonised than the plastic surfaces, having a maximum of 3.50×10^5 cfu cm⁻², significantly lower than that on the plastic materials (at a 95% confidence limit). No legionella were detected in any of the biofilms sampled on copper (limit of detection was 10 cfu cm⁻²). The diversity of microorganism present on the copper surface was significantly reduced when compared to the polybutylene, with only 4 species of pseudomonads and two other genera of bacteria present on the copper surface (Table 5.4). *Pseudomonas paucimobilis* was the most abundant pioneer, with *P. mendocina*, *P. testosteroni* and *P. xylesoxidans* also being detectable. *Pseudomonas paucimobilis* remained dominant in the climax community, which had increased in diversity due to the inclusion of *P. testosteroni* and *Flavobacterium* sp. Although protozoa were detectable within the culture and on the glass surface in the presence of copper at 20 °C none appeared on the surface of the copper tile over the 21 day experiment.

Glass control surfaces: Colonisation of the glass control surfaces reflected that on the materials themselves. The glass surface that had been suspended alongside copper tube was least colonised (Table 5.2) and had no detectable *L. pneumophila* for the duration of the colonisation experiment (Figure 5.2), indicating that the copper was inhibiting colonisation of the glass surface. The plastics supported higher biofouling than their glass control surfaces but the numbers of *L. pneumophila* were slightly lower on PVCc than on the glass control, however, this was not significantly different.

Figure 5.1 Total non-legionella flora colonising the surface of copper (▲), polybutylene (●) and PVCc (▼) at 20°C.

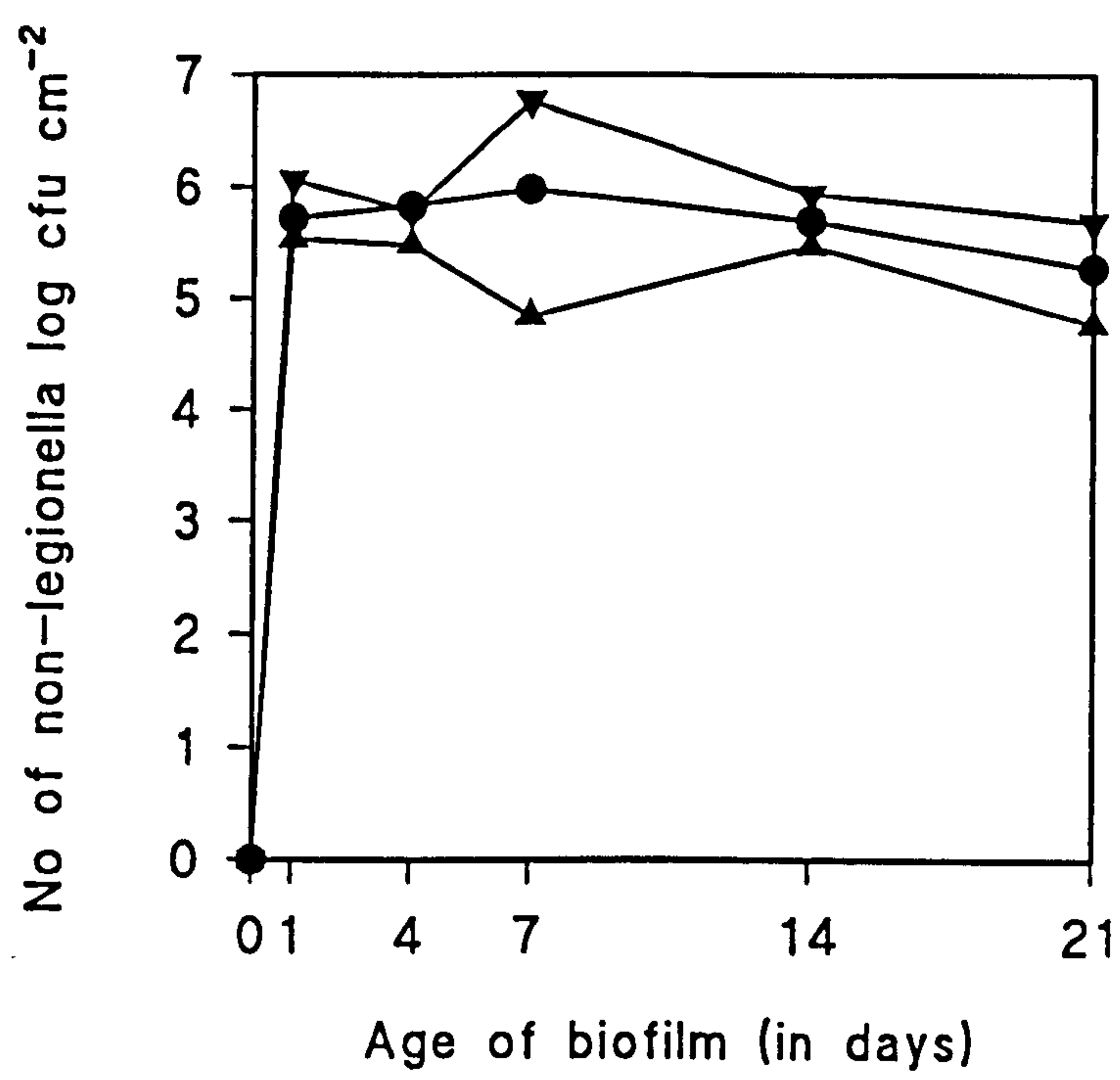


Figure 5.2 Numbers of *L. pneumophila* colonising the surface of copper (▲), polybutylene (●) and PVCc (▼) at 20°C.

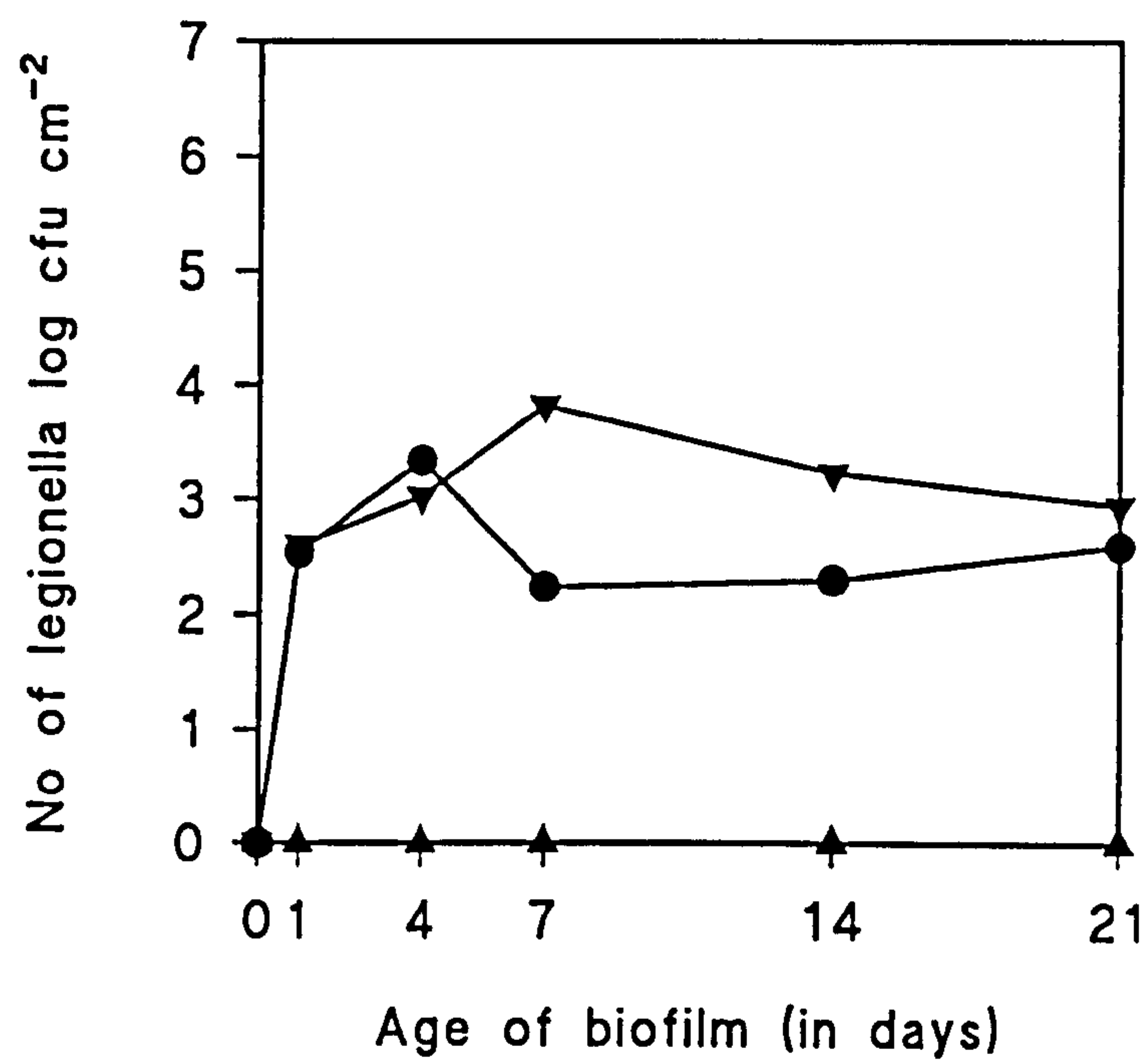


Table 5.3 Comparison of the colonisation of different plumbing materials and inclusion by *L. pneumophila* at different temperatures.

Material	Mean Colonisation (cfu/cm ²)		colonisation ratio	
	Total flora	<i>L. pneumophila</i>	Total flora	<i>L. pneumophila</i>
20°C				
Copper	2.16x10 ⁵	0	1	-
Polybutylene	5.70x10 ⁵	665	2.6	665
PVCc	1.81x10 ⁶	2132	8.4	2132
40°C				
copper	8.04x10 ⁴	1967	1	1
Polybutylene	1.18x10 ⁶	111880	14.7	56.8
PVCc	3.67x10 ⁵	68379	4.6	34.7
50°C				
copper	2.26x10 ⁴	0	1	1
Polybutylene	3.21x10 ⁶	868	142	868
PVCc	1.22x10 ⁵	60	5.4	60
60°C				
Copper	4.47x10 ²	0	1	-
Polybutylene	4.25x10 ⁴	0	95	-
PVCc	5.19x10 ³	0	11.6	-

The colonisation ratio is the total non-legionella or legionella population recovered from each material when compared with the copper data. Means were calculated from all values determined over 1-21 days.

Table 5.4 Communities of microorganisms which colonise the surface of copper

	Pioneering microorganisms cfu/cm ² (x10 ⁻³)					Climax community of microorganisms cfu/cm ² (x10 ⁻³)				
	20°C	40°C	50°C	60°C	60°C	20°C	40°C	50°C	60°C	60°C
L. pneumophila	< 0.01	0.02	< 0.01	< 0.01	< 0.01	< 0.01	0.09	< 0.01	< 0.01	< 0.01
P. aeruginosa	80					8				
P. mendocina	120	41	0.6	0.6		660	1.3			
P. paucimobilis						7				
P. testosteroni	80					6	0.2			
P. xylosoxidans										
Actinomycetes		2								
Flavobacterium						10	12			
Methylobacterium			20	0.7			3		0.12	
Alcaligenes		10								
Aspergillus		0.5					0.01			
P. hydrogenophaga			12.5	2.7				72		0.8

Numbers of non-legionella populations are represented as a sum of those cfu cm⁻² occurring on R2A, BCYE and GVPC media. The limit of detection of *L. pneumophila* was 10 cfu cm⁻².

Table 5.5 Communities of microorganisms which colonise the surface of polybutylene.

	Pioneering microorganisms					Climax community of microorganisms				
	cfu/cm ² (x10 ⁻³)					cfu/cm ² (x10 ⁻³)				
	20°C	40°C	50°C	60°C	20°C	40°C	50°C	60°C		
<i>L. pneumophila</i>	3.6	0.6	0.15	<0.01	0.4	63	0.4			<0.01
<i>P. aeruginosa</i>		300	0.4		8	130				
<i>P. fluorescens</i>					20	200				
<i>P. maltophilia</i>	10									
<i>P. mendocina</i>	10	200			0.2	200				
<i>P. paucimobilis</i>	100	200	0.3		630					13
<i>P. stutzeri</i>	13				0.6	160				
<i>P. testosteroni</i>	340	1200			160					
<i>P. vesicularis</i>		600			163					
<i>P. xyloxydans</i>		1500								
<i>Actinomyces</i>	120				40					
<i>Flavobacterium</i>		100				31			2	
<i>Methylobacterium</i>		10		0.17		10	30		6	
<i>Alcaligenes</i>		1200								
<i>Aspergillus</i>						4	0.4		1.6	
<i>P. hydrogenophaga</i>			6600	3.9			5600		58	
<i>Acinetobacter</i>	10	1000				480				
Unidentified Gram-negatives		200		0.06					6	

Numbers of non-legionella populations are represented as the sum of these cfu cm⁻² occurring on R2A, BCYE and GVPC media. The limit of detection of *L. pneumophila* as 10 cfu cm⁻².

5.3.3 Growth in biofilms at 40°C.

Polybutylene: Polybutylene was the most rapidly colonised surface at 40°C with 1.55×10^6 cfu cm⁻² after 24h, increasing to 3.25×10^6 cfu cm⁻² after 14 days. *Legionella pneumophila* were also most readily incorporated into the biofilm forming on this surface, with 6.00×10^2 cfu cm⁻² after 24h. The pioneers were a diverse mixture of microorganisms composed of 62% pseudomonads and 38% other Gram negative organisms. The pseudomonads were dominated by *P. testosteroni* and *P. xyloxydans* with other species including *P. aeruginosa*, *P. mendocina*, *P. paucimobilis* and *P. vesicularis*. *Alcaligenes* sp., *Flavobacterium* sp., *Acinetobacter* sp. and one further gram negative organisms that would not grow after subculture were also present. Numbers of the pathogen increased to 3.78×10^5 cfu cm⁻² after 14 days, accounting for 48% of the total biofilm flora. *Alcaligenes* sp., *P. xyloxydans*, *P. vesicularis*, *P. paucimobilis* were replaced in the climax community by *P. fluorescens*, *P. mendocina*, *P. stutzeri* and *Aspergillus* sp. The amoebae and other protozoa which had been clearly evident at 20°C were not detectable at 40°C.

PVCc: The PVCc surface was also rapidly colonised by the mixed consortia of microorganisms, but at a significantly lower level than that on the polybutylene surface. The biofilm on PVCc contained a total non-legionella population of between $1.53 - 7.40 \times 10^5$ cfu cm⁻². The biofilm on the PVCc surface supported a high population of *L. pneumophila* at 40°C with 7.40×10^5 cfu cm⁻² after 4 days when the pathogen was 35% of the biofilm flora. There was no significant difference between the numbers of *L. pneumophila* on the plastic surfaces.

Copper: Copper was the least colonised of the surfaces having a maximum of 5.05×10^4 cfu cm⁻² bacteria and this was significantly lower than that on the plastic surfaces. Copper surfaces were however, capable of sustaining *L. pneumophila* in biofilms after

24h, but the numbers of the pathogen were significantly less than those on the surface of either plastic. The population on the copper surface was initially dominated by *P. paucimobilis* with the other microorganisms occurring in low numbers. *Legionella pneumophila* (2.00×10^2 cfu cm⁻²), *Methylobacterium* sp., *Alcaligenes* sp., *Aspergillus* sp. and actinomycetes were the only other detectable bacteria. The climax community supported *Flavobacterium* sp. and *Aspergillus* sp. and had a total *L. pneumophila* population of 9.00×10^3 cfu cm⁻² (29% of the biofilm flora).

Glass control surfaces: Glass tiles suspended in the presence of polybutylene were rapidly colonised by the non-legionella population to form a biofilm that contained 7.9×10^5 cfu cm⁻² after 24h and had a mean colonisation of 4.43×10^5 (table 2). The biofilm supported a *L. pneumophila* population of 4.05×10^5 cfu cm⁻² after 7 days and the pathogen accounted for 14% of total biofilm flora.

The glass tile immersed with PVCc also supported a high bacterial flora with between 1.07 - 2.10×10^5 cfu cm⁻² over the 21 day period and *L. pneumophila* accounted for up to 10% of total biofilm flora (1.2×10^5 cfu cm⁻² after 4 days).

Copper reduced the colonisation of glass tiles suspended in its presence, with biofilms developed less rapidly (5.85×10^4 cfu cm⁻² after 24 h). For the duration of the experiment there was a total non-legionella population of 3.35 - 3.80×10^4 cfu cm⁻², with the exception of the maximum of 1.45×10^5 cfu cm⁻² which occurred on the fourth day. The presence of copper also suppressed the inclusion of *L. pneumophila* into the biofilm on the control glass surface with numbers of the pathogen being between 2.35×10^2 and 1.44×10^3 cfu cm⁻². Both the total biofouling and the numbers of *L. pneumophila* was significantly lower on copper than on the plastic surfaces.

Figure 5.3 Total non-legionella flora colonising the surfaces of copper (▲), polybutylene (●) and PVCc (▼) at 40°C.

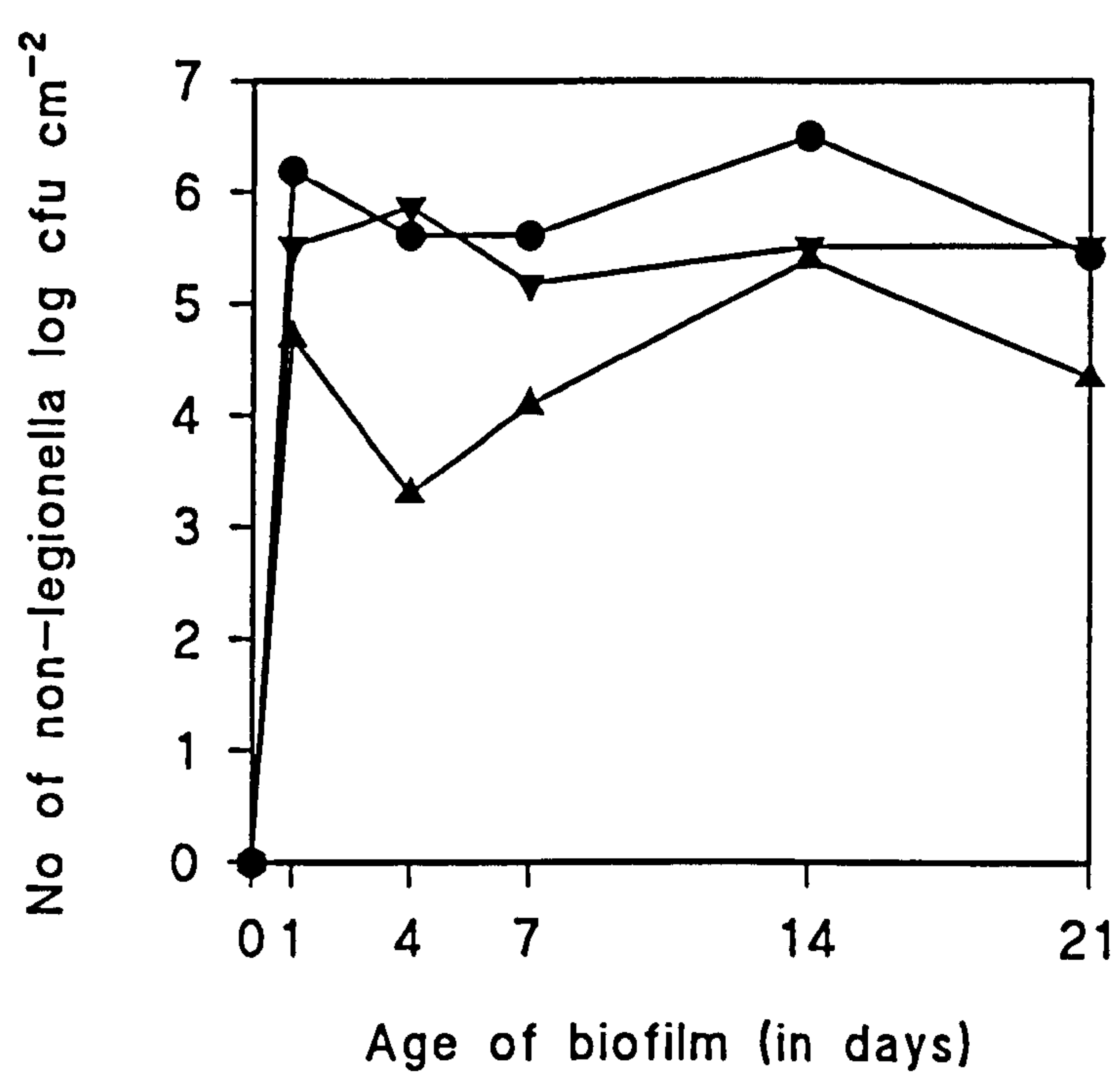
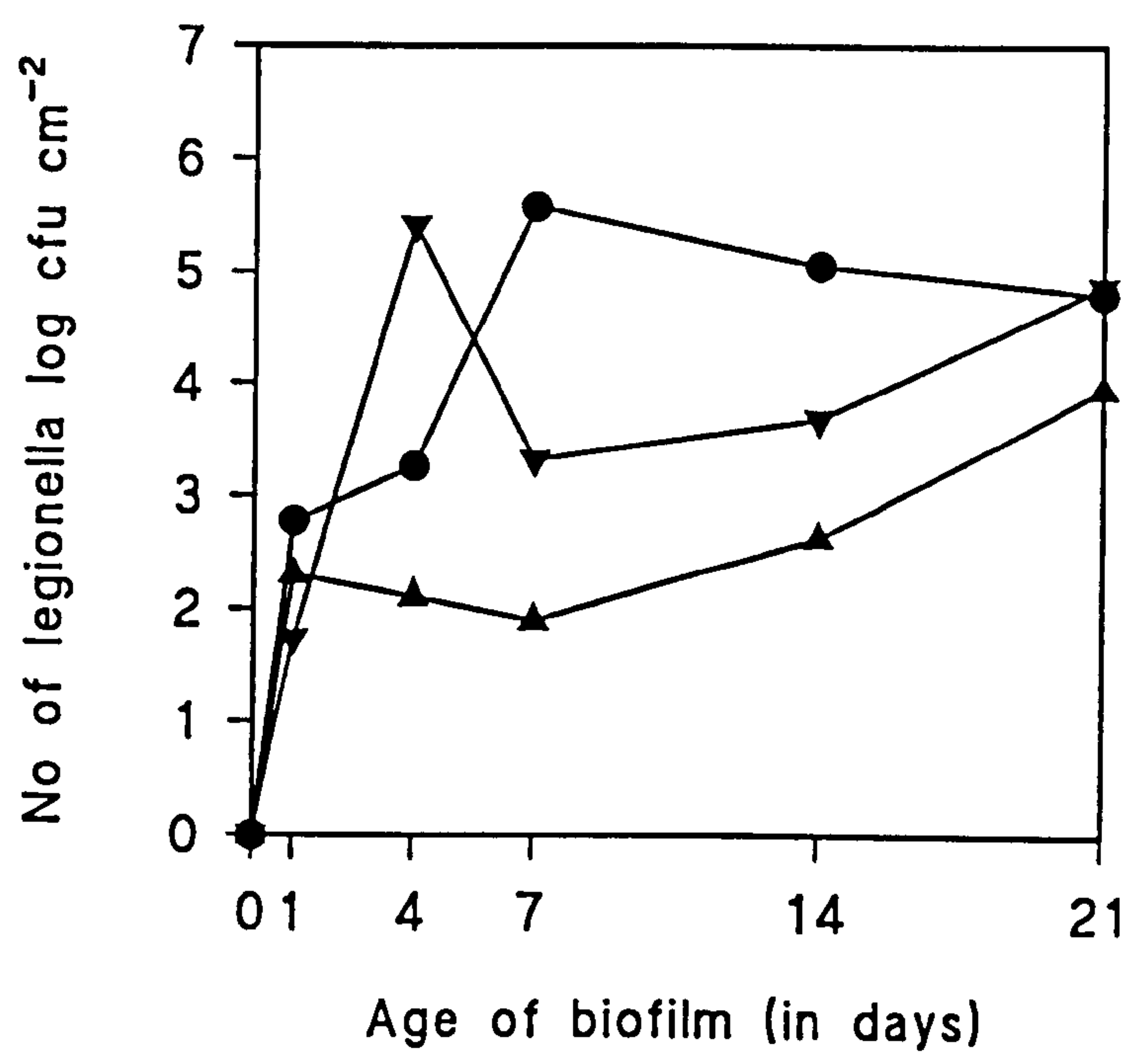


Figure 5.4 Numbers of *L. pneumophila* colonising the surface of copper (▲), polybutylene (●) and PVCc (▼) at 40°C.



5.3.4 Growth in biofilms at 50°C.

Polybutylene: Polybutylene was rapidly colonised at 50°C forming a biofilm containing 1.55×10^6 cfu cm⁻² microorganisms after only 24h and this biofilm contained low numbers of *Legionella pneumophila* (1.50×10^2 cfu cm⁻²). The diversity of the microbial population was greatly reduced at 50°C with the only pioneers being pseudomonads, these were *P. aeruginosa*, *P. paucimobilis* and the predominant *P. hydrogenophaga*. The high microbial population was maintained throughout the experiment (2.05 - 3.45×10^6 cfu cm⁻²) but *L. pneumophila* numbers remained low, reaching a maximum of 3.55×10^3 cfu cm⁻² after 21 days and accounting for 0.1 % of the biofilm flora. The only other microorganisms within the climax community were *P. hydrogenophaga* and *A. fumigata*, both of which could be cultured on agar plates at 50°C. No thermotolerant protozoa were detected by either light microscopy or culture.

PVCc: The PVCc was rapidly colonised by non-legionella populations (8.15×10^4 cfu cm⁻²) but numbers were consistently lower than on polybutylene and this was statistically significant. Although *L. pneumophila* was not incorporated into biofilms until day 7, the pathogen was present within the climax community at a concentration of 2.60×10^2 cfu cm⁻².

Copper: Copper supported a sparse biofilm with only 7.13×10^3 cfu cm⁻² after 24 h, this gradually increased over the 21 day trial to 5.60×10^4 cfu cm⁻² which was significantly lower than those on PVCc and polybutylene. No *L. pneumophila* were detectable on the copper surface over the duration of the experiment. *Methylobacterium sp.* were the predominant pioneers accounting for 62% of the total biofilm flora, with *P. hydrogenophaga* and *P. paucimobilis* also being present. *Pseudomonas hydrogenophaga* became increasing abundant and represented 99% of the climax

community.

Glass control surfaces: Glass incubated with polybutylene was rapidly colonised by microorganisms (2.70×10^6 cfu cm⁻² after only 24h) and numbers increased to a maximum of 3.95×10^6 cfu cm⁻². Low numbers of *L. pneumophila* were present in these biofilms with a maximum of 1.30×10^3 cfu cm⁻² after 21 days.

Glass incubated with PVCc was less colonised than that with polybutylene (9.00×10^4 cfu cm⁻² after 24h). A maximum of 1.46×10^6 cfu cm⁻² non-legionella occurred at 21 days. *Legionella pneumophila* was present on the glass surface incubated with PVCc, at a concentration of 3.50×10^1 cfu cm⁻² after 14 days increasing to 1.80×10^2 cfu cm⁻² after 21 days.

Copper suppressed colonisation of glass tiles incubated alongside, with only 7.80×10^3 cfu cm⁻² total biofilm flora after 24h. Although the biofouling increased to a maximum of 4.30×10^5 cfu cm⁻² after 14 days, this was lower than that occurring on the glass surfaces incubated with the plastics. *Legionella pneumophila* was only detectable on one occasion in the biofilms on glass incubated with copper, and this was at the limit of detection (1.0×10^1 cfu cm⁻²).

Figure 5.5 Total non-legionella flora colonising the surfaces of copper (▲), polybutylene (●) and PVCc (▼) at 50°C.

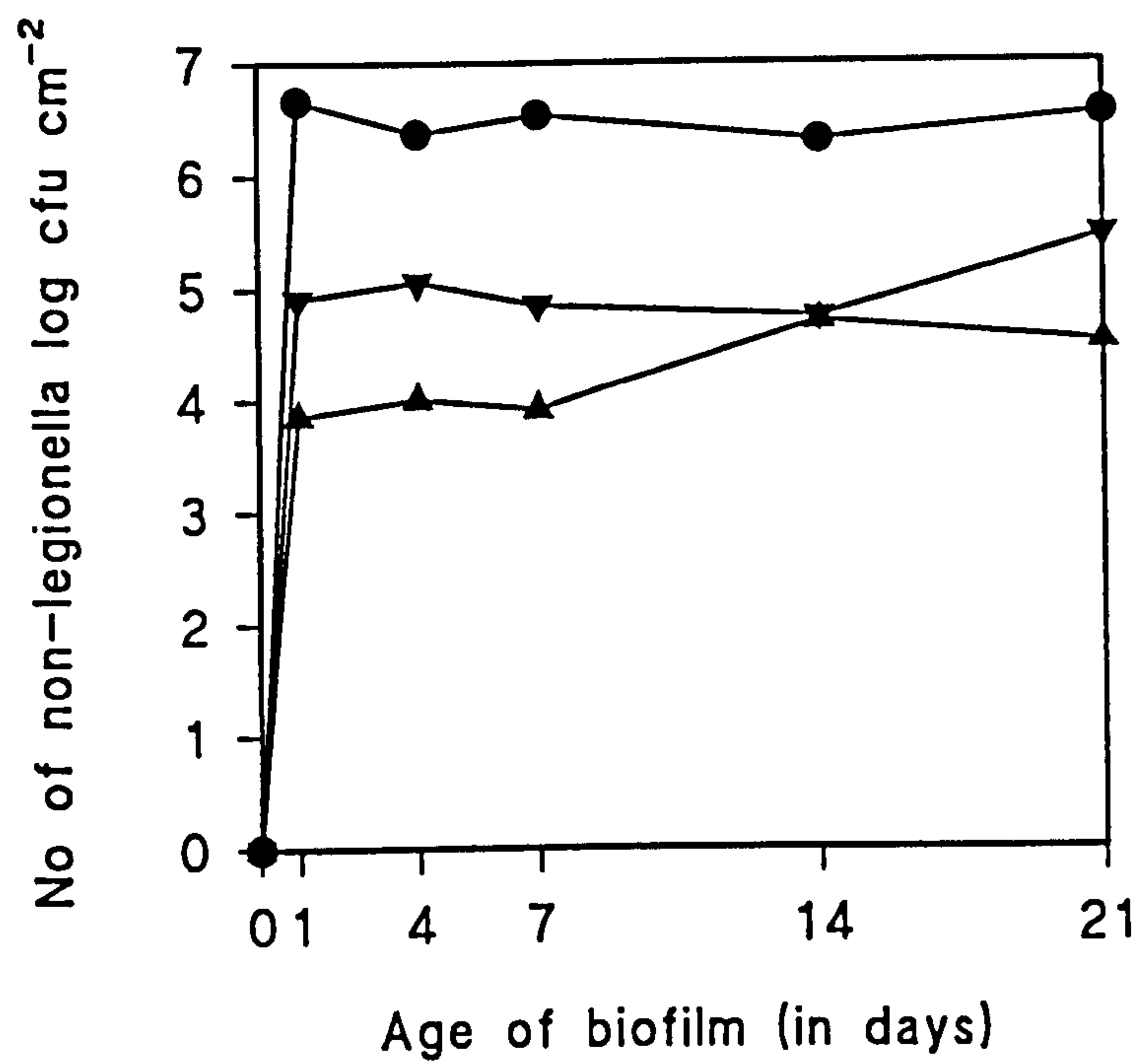
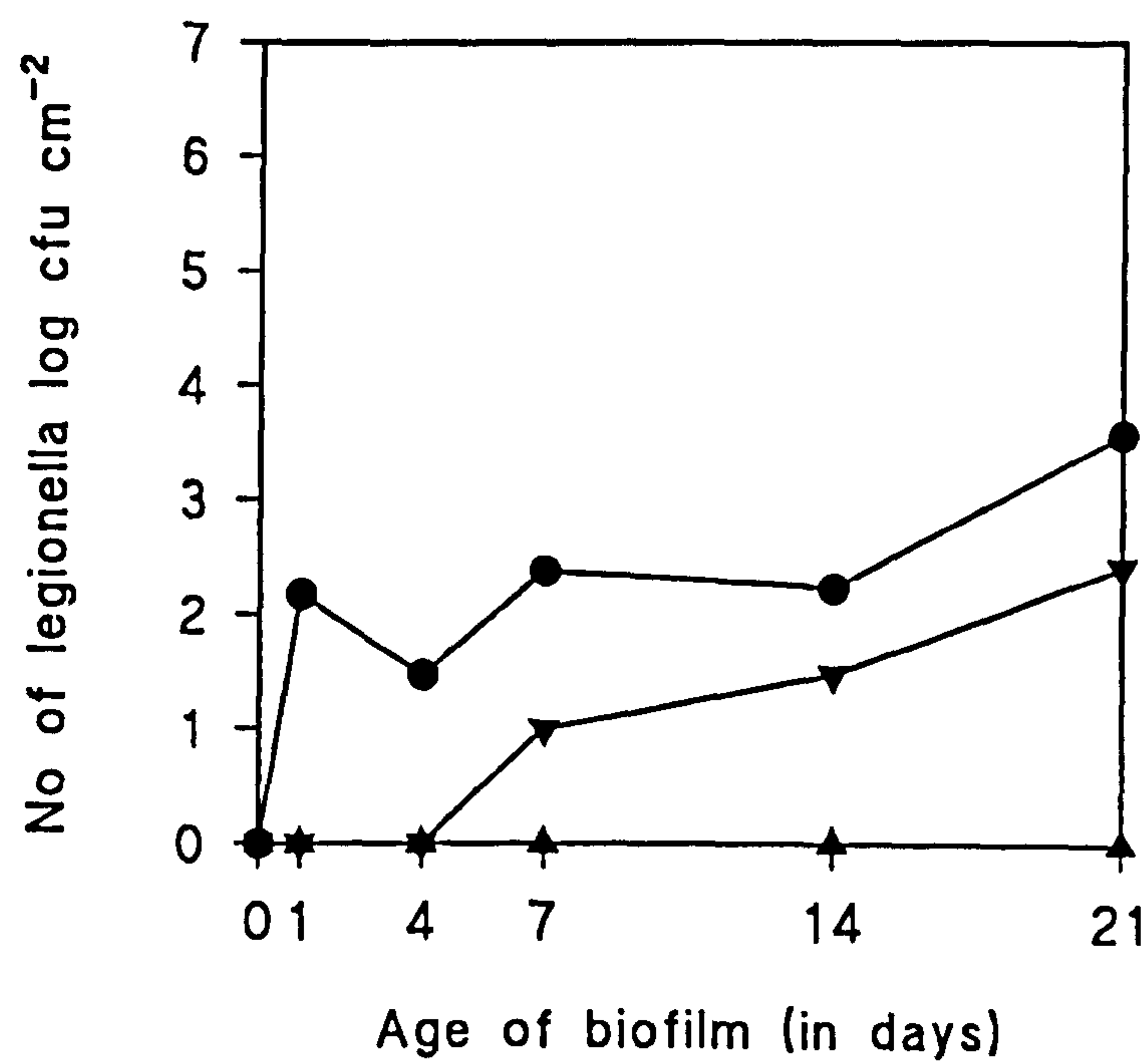


Figure 5.6 Numbers of *L. pneumophila* colonising the surface of copper (▲), polybutylene (●) and PVCc (▼) at 50°C.



5.3.5 Growth in the biofilms at 60°C.

Polybutylene: Polybutylene was the most rapidly colonised surface at 60°C supporting a total non-legionella population of 5.35×10^3 cfu cm⁻² after 24h and numbers increased to a maximum of 4.95×10^4 cfu cm⁻². *Legionella pneumophila* was not detected in biofilms on polybutylene at 60°C. The biofilm developing on polybutylene were dominated by *P. hydrogenophaga* and *A. fumigata*. There were several other species transiently present, including *P. paucimobilis*, *Flavobacterium* sp. and *Methylobacterium* sp. but their low numbers and erratic occurrence indicated that they were survivors from the first chemostat rather than active members of the community.

PVCc: The biofilm developing on PVCc supported a total flora of 9.40×10^2 cfu cm⁻² and this increased to a maximum of 1.33×10^4 cfu cm⁻² after 14 days. *Legionella pneumophila* was not detected in any sample at 60°C.

Copper: Copper was least colonised of the materials tested, significantly less than the plastic materials. After 24 h the biofilm contained 2.75×10^2 cfu cm⁻², increasing only slightly to 8.50×10^2 cfu cm⁻² after 14 days. The sparse biofilms were principally composed of *P. hydrogenophaga* with *P. paucimobilis* and *Methylobacterium* sp. also present at 24h. The climax community contained only *P. hydrogenophaga*. *Legionella pneumophila* could not be detected.

Glass control surfaces: In the presence of polybutylene the glass surface supported less total biofilm flora than the plastic material. After 24h the total biofilm flora was 2.85×10^3 cfu cm⁻² increasing to 3.80×10^4 cfu cm⁻² after 21 days. Glass tiles incubated with PVCc also supported a sparse biofilm at 60 °C with a total biofilm population of 1.33×10^3 cfu cm⁻² after 24h which increased to a 5.45×10^4 cfu cm⁻² after 14 days.

Copper inhibited colonisation of glass with only 4.00×10^2 cfu cm⁻² after 24h and numbers did not increase above 8.80×10^2 cfu cm⁻². *Legionella pneumophila* was not detected in any of the biofilms on the glass surfaces at 60°C.

Figure 5.7 Total non-legionella flora colonisation the surfaces of copper (▲), polybutylene (●) and PVCc (▼) at 60°C.

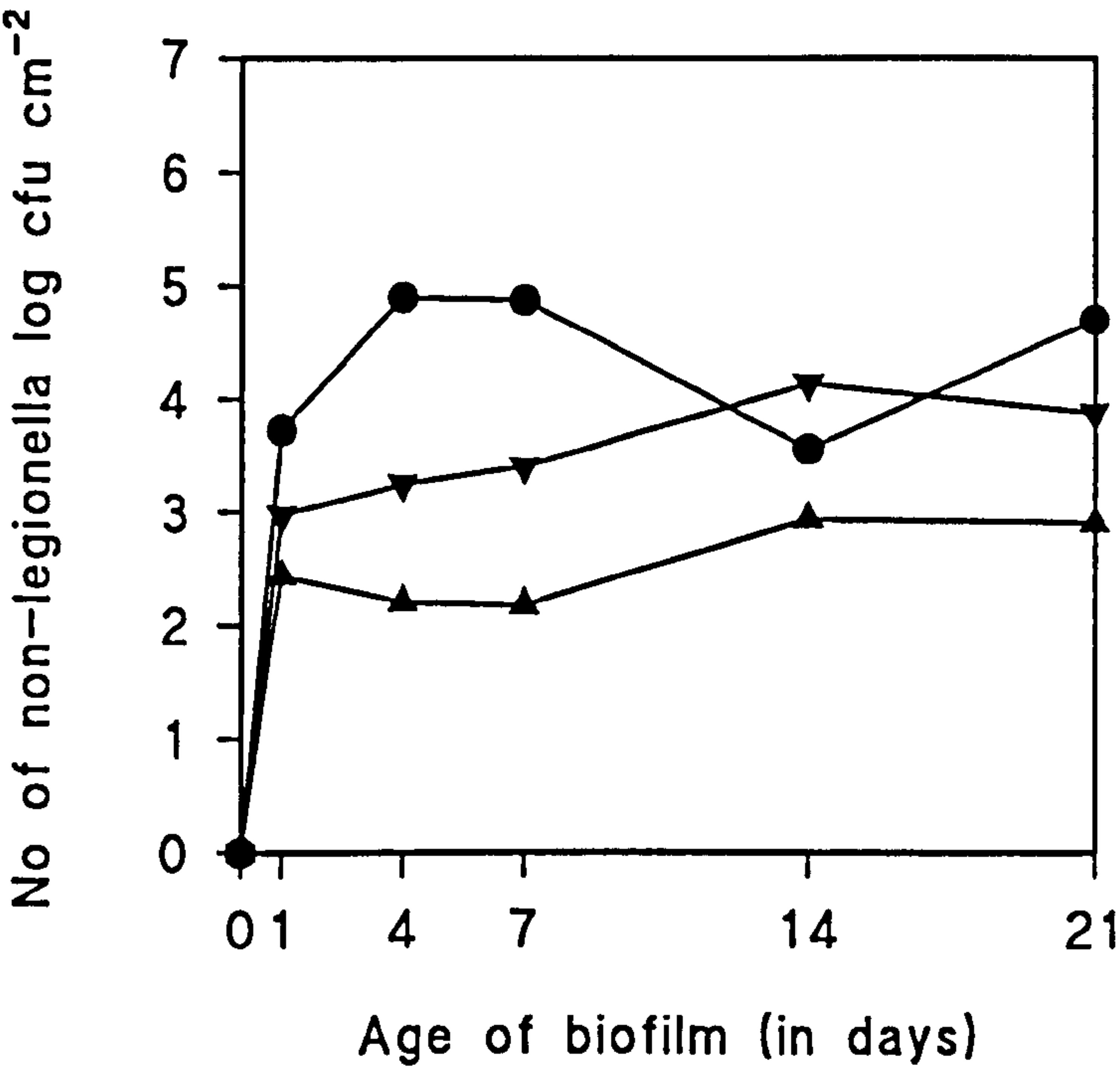
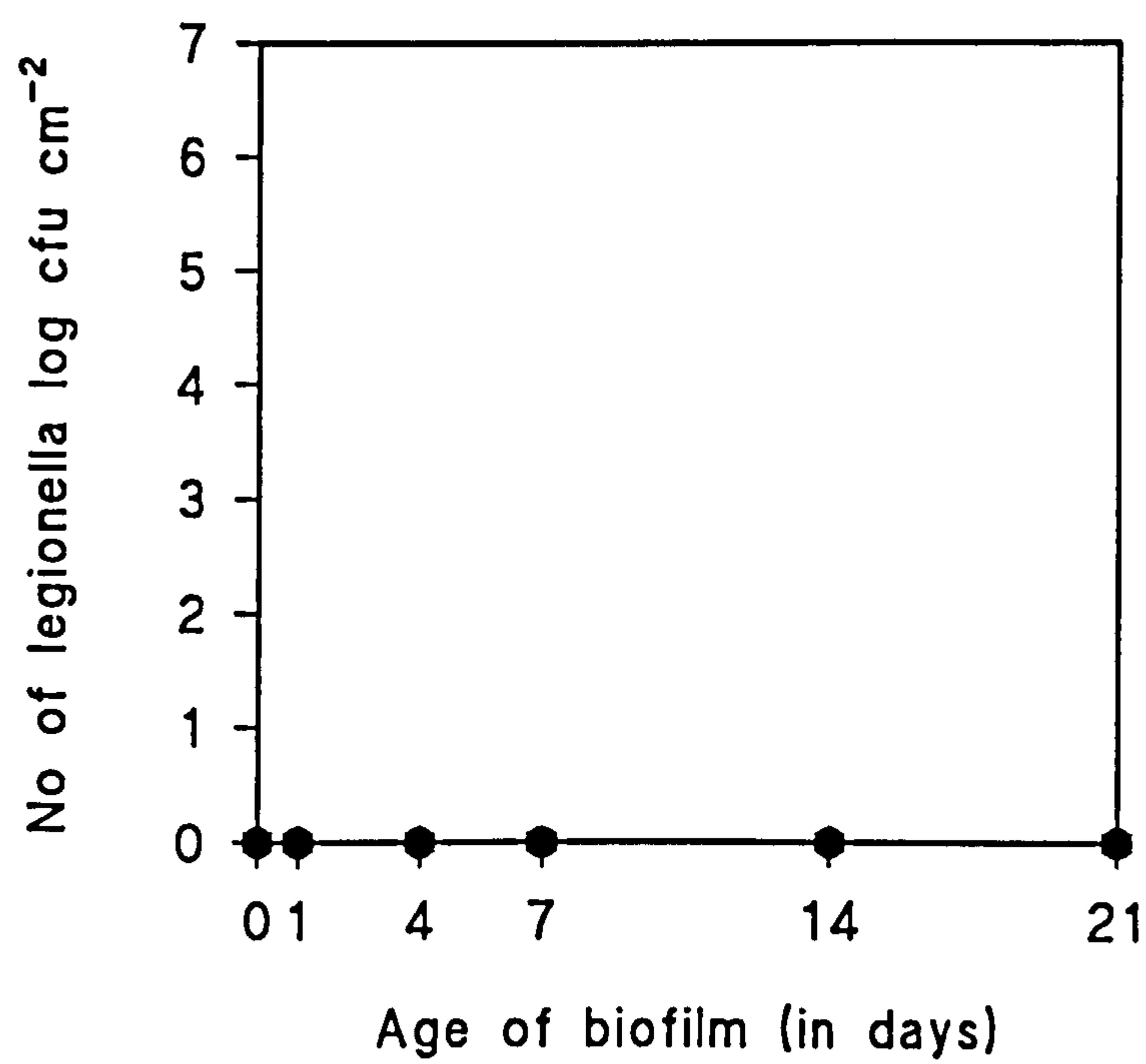


Figure 5.8 Numbers of *L. pneumophila* colonisation the surface of copper (▲), polybutylene (●) and PVCc (▼) at 60°C.



5.3.6 Microscopy of biofilm formation.

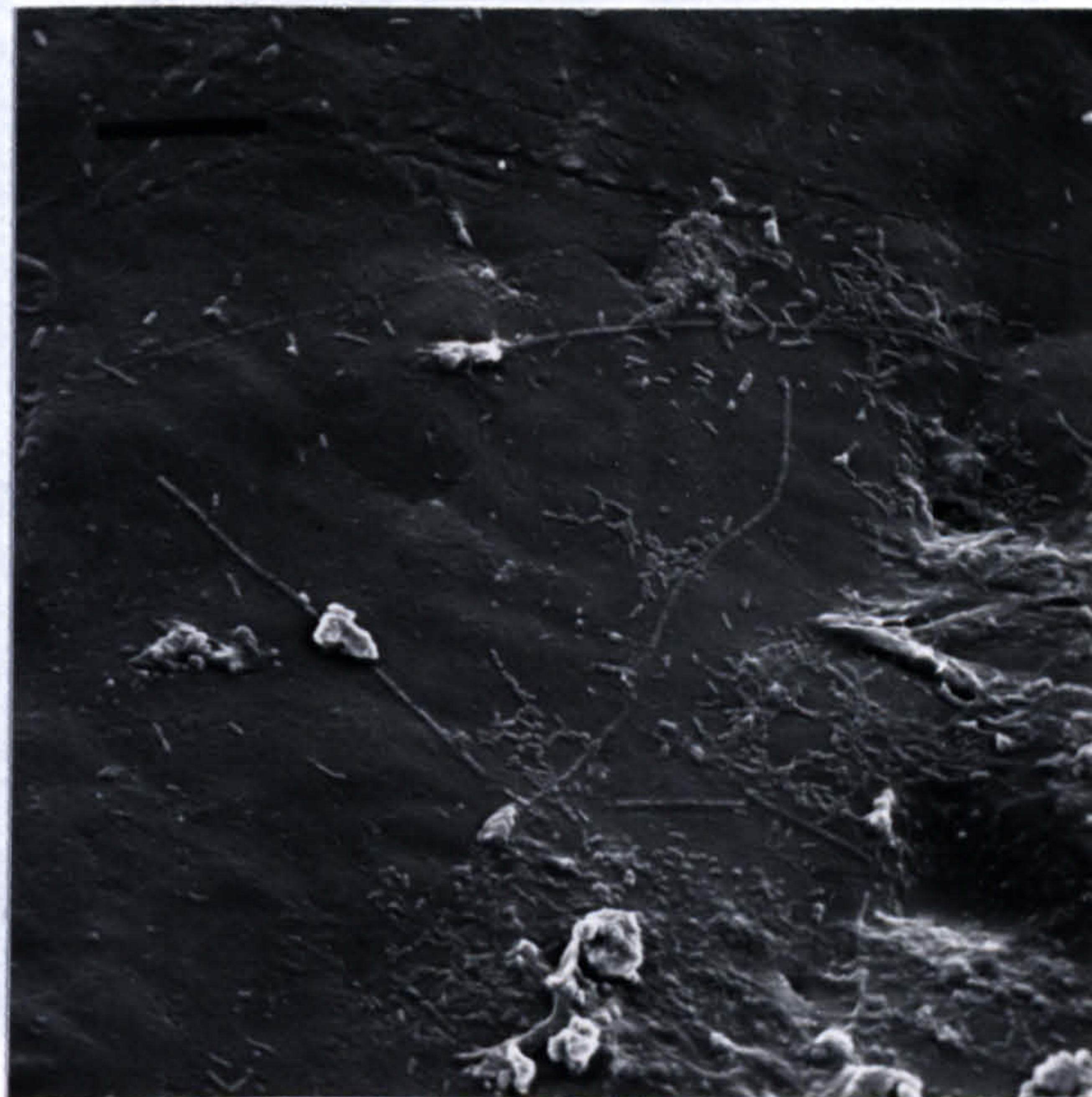
At 20°C: There was no evidence of bacterial colonisation of copper at 20°C due to the crystalline nature of the copper surface.

Bacteria had formed microcolonies in isolated areas on the polybutylene surface after only 1 day in the chemostat model (Figure 5.9a). Polymer could be observed around the bacterial cells, most of which were short rods. Bacteria could be observed embedded within extracellular polymer which covered the whole surface of the tile (Figure 5.9b). By day 21 the biofilm on the polybutylene surface was extensive with cells being evident in much deeper biofilm. The thickness of the biofilm is indicated by the cracks which developed on drying. Cells could be observed embedded within extracellular polymer (Figure 5.9c). After 28 days immersion into the chemostat culture the surface of the polybutylene was completely covered by a thick layer of biofilm (Figure 5.9d). Long chains of bacteria could be distinguished within the polymer but most of the bacterial cells could not be observed due to the large amount of extracellular material surrounding them.

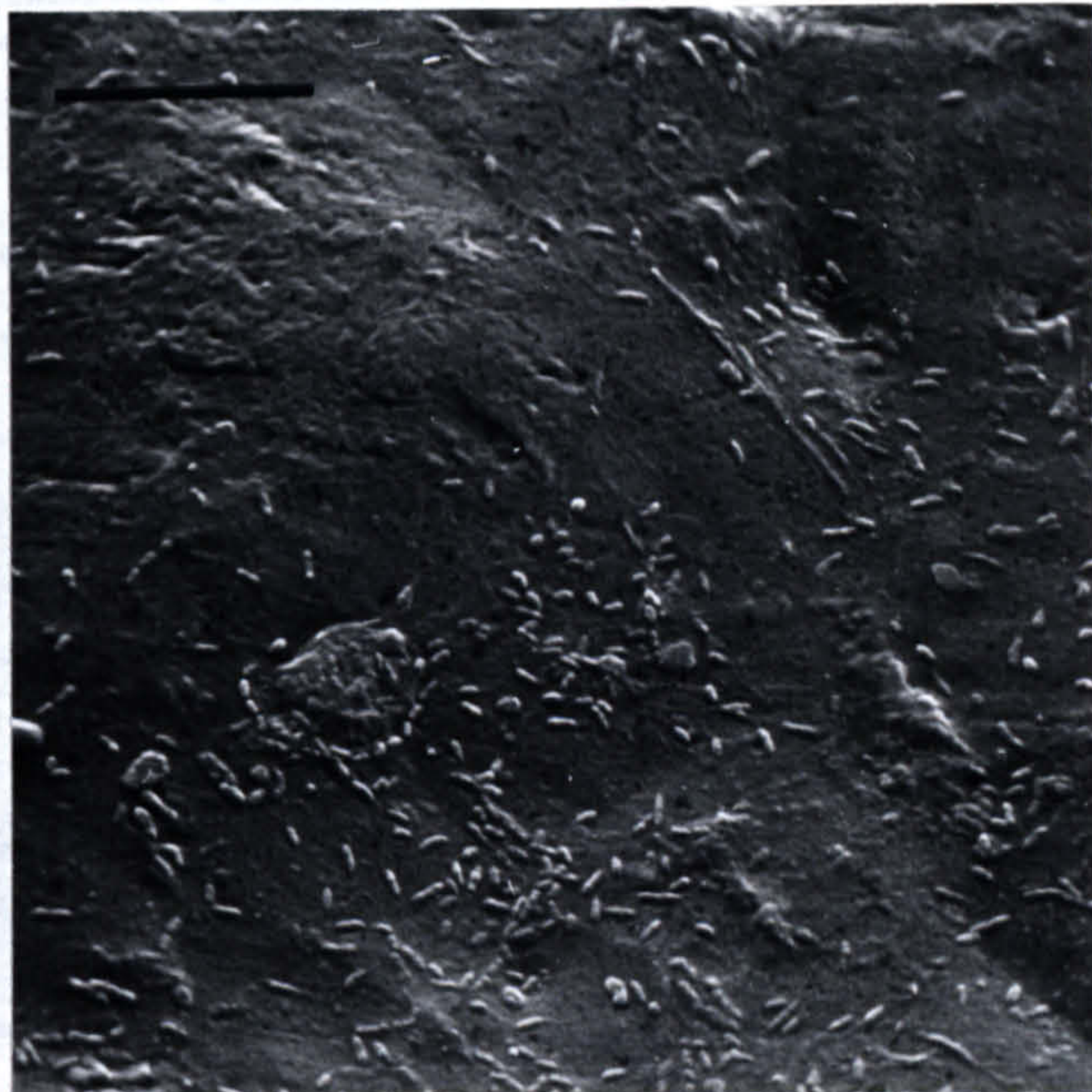
At 40°C: Areas of biofilm formed on polybutylene where bacterial cells could be observed on the surface after only 24 h and these areas were associated with extracellular polymer (Figure 5.10a). After 14 days biofilm appeared to be covered in a thick layer of amorphous material (Figure 5.10b and c). The biofilm was extensive after 21 days and polymer was a substantial component. The bacterial cells could be observed embedded within the extracellular matrix and recolonising the surface (Figure 5.10d).

Figure 5.9 Scanning electron micrographs of biofilms developing on polybutylene after 1 (a), 4 (b), 21 (c) and 28 (d) days at 20°C.

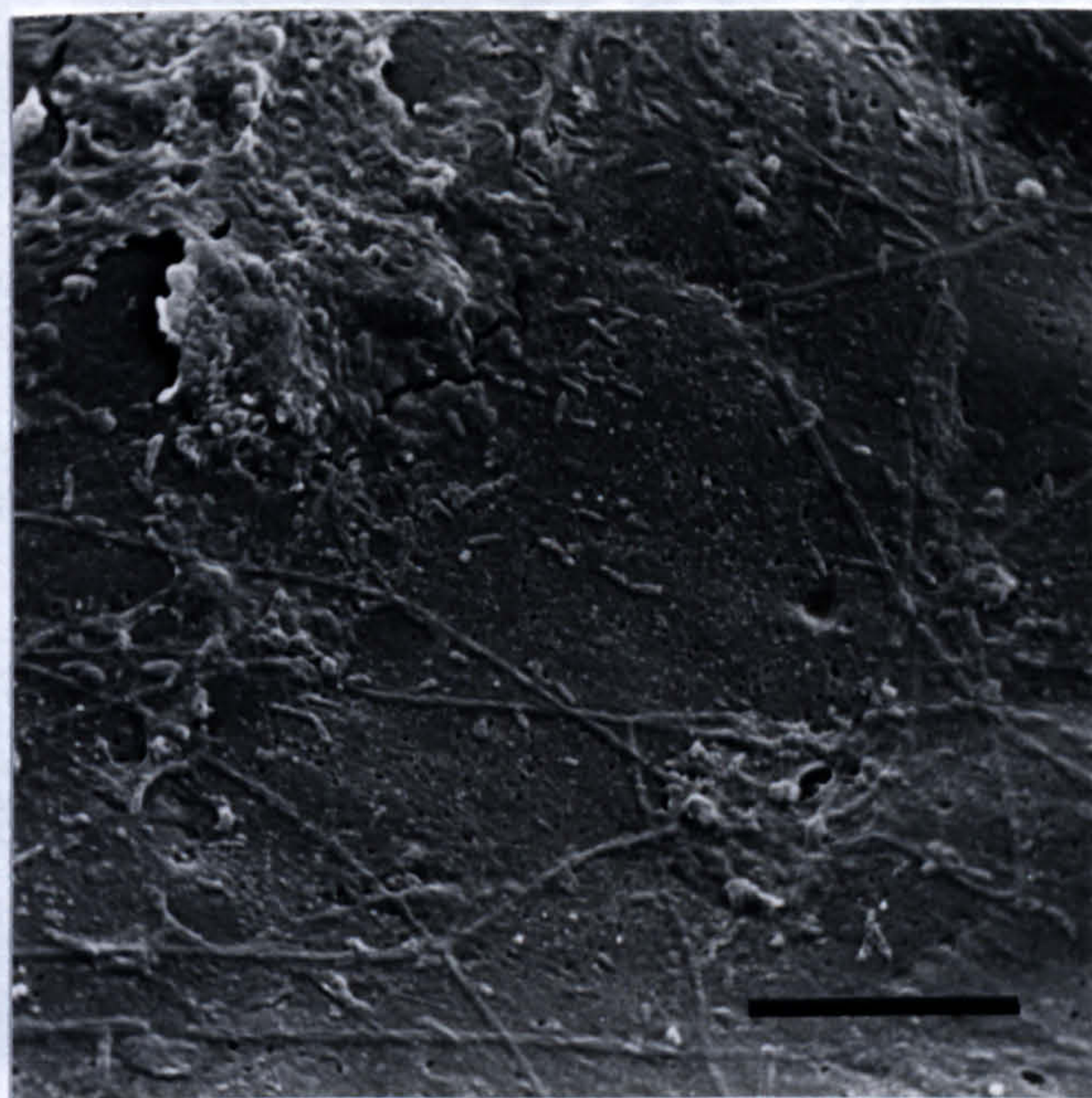
(a)



(b)



(c)



(d)

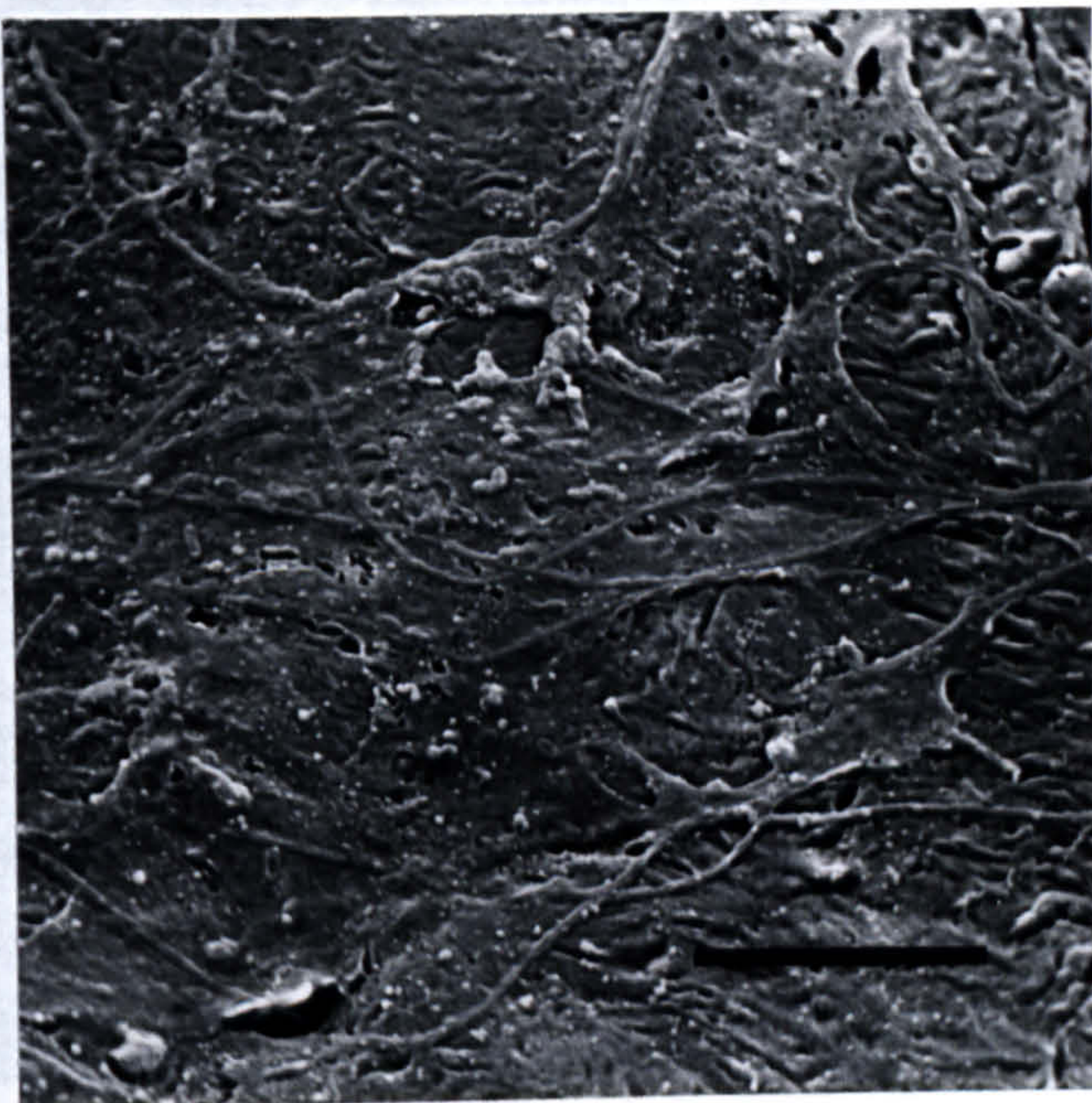
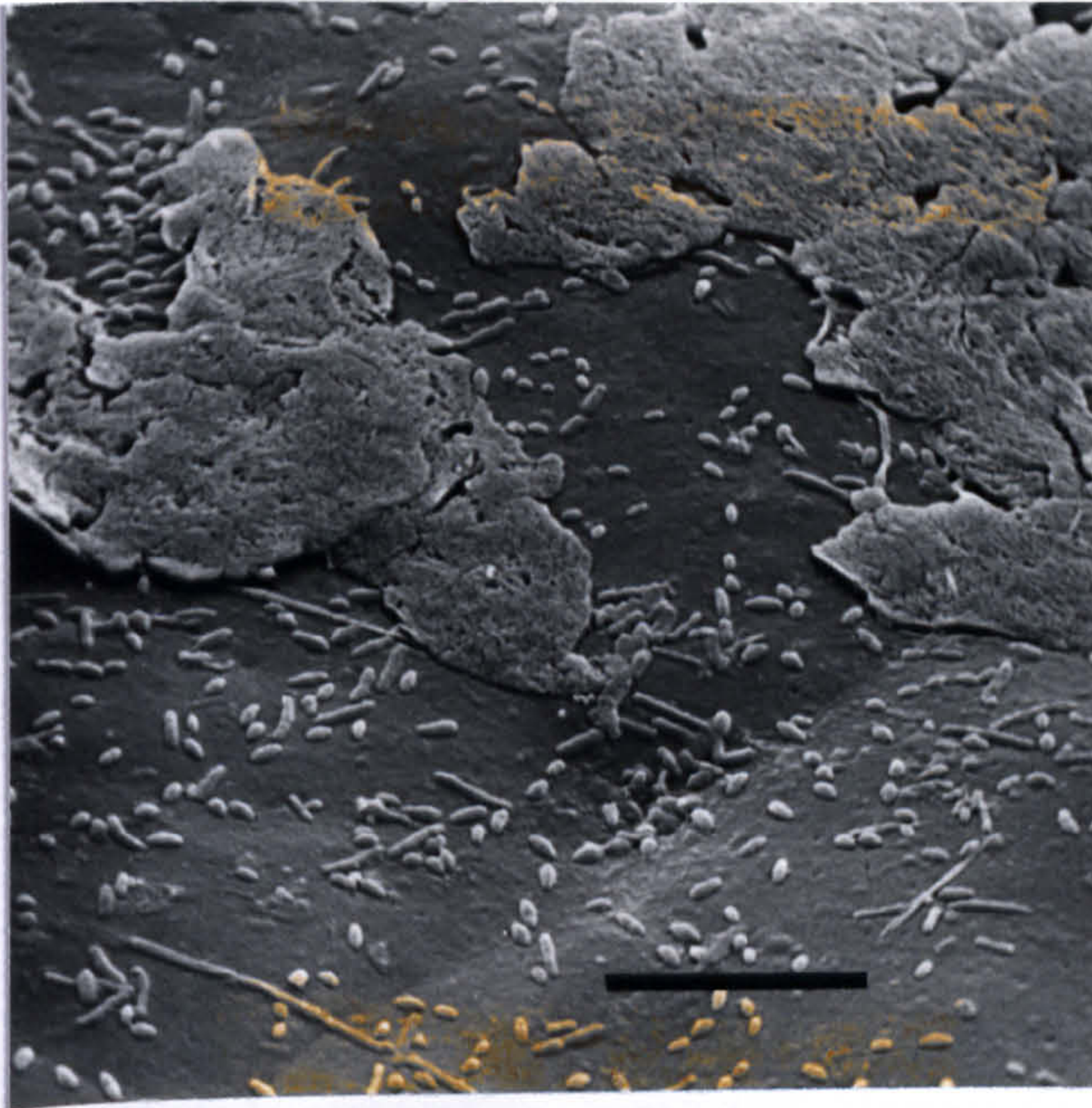
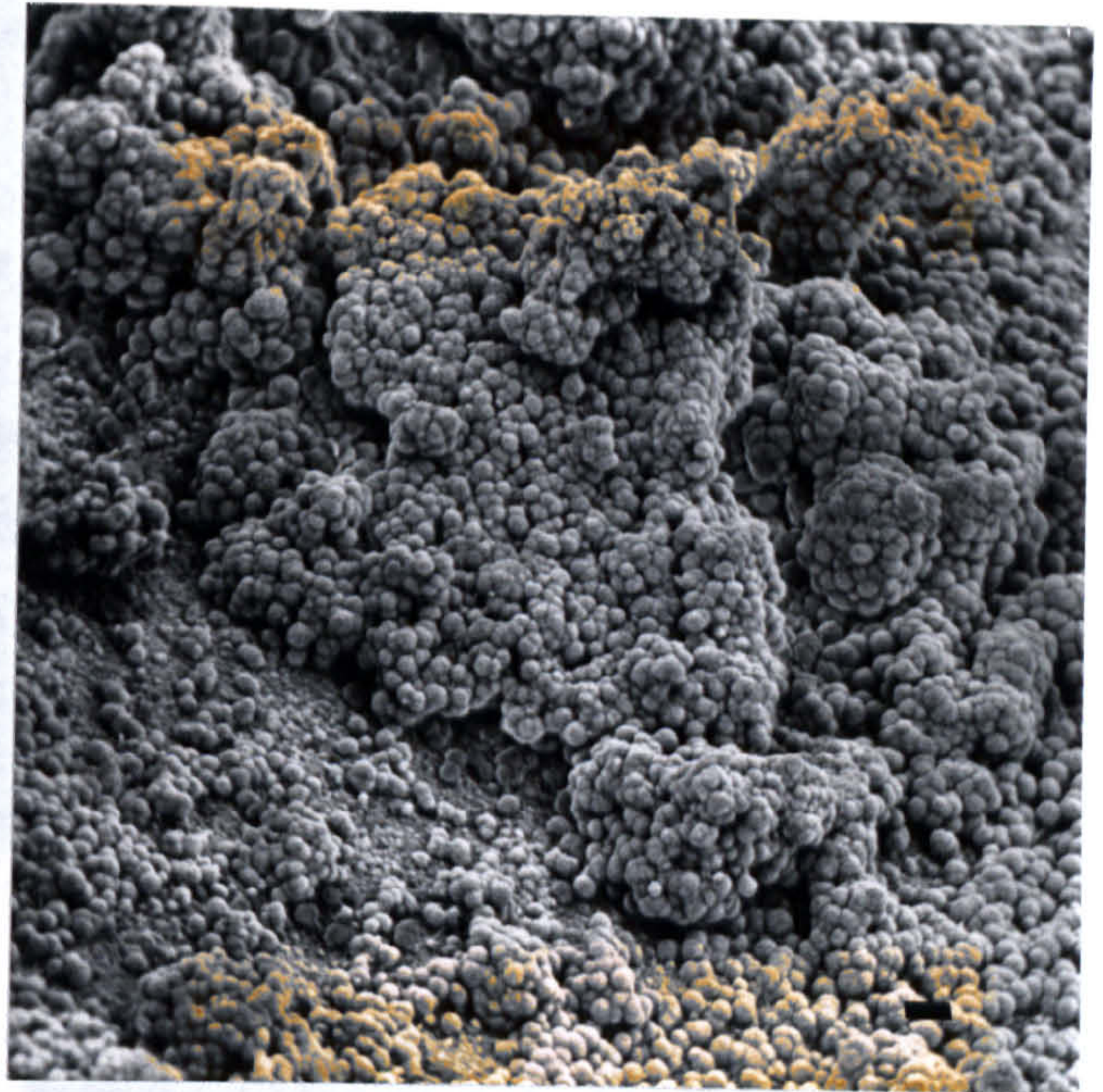


Figure 5.10 Scanning electron micrographs of biofilm formation on the surface of polybutylene after 1(a), 14 (b and c) and 28 (d) days at 40°C.

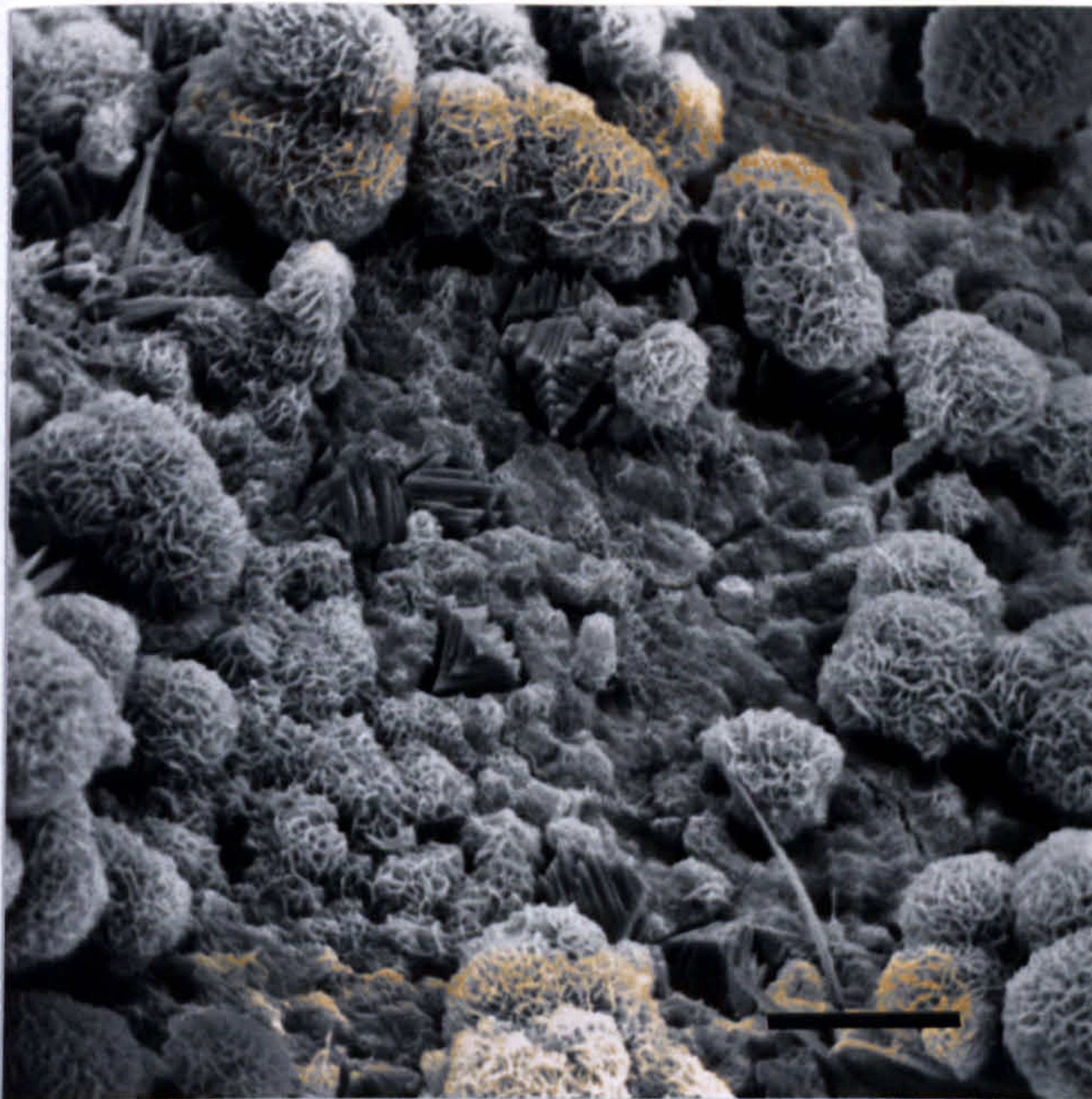
(a)



(b)



(c)



(d)

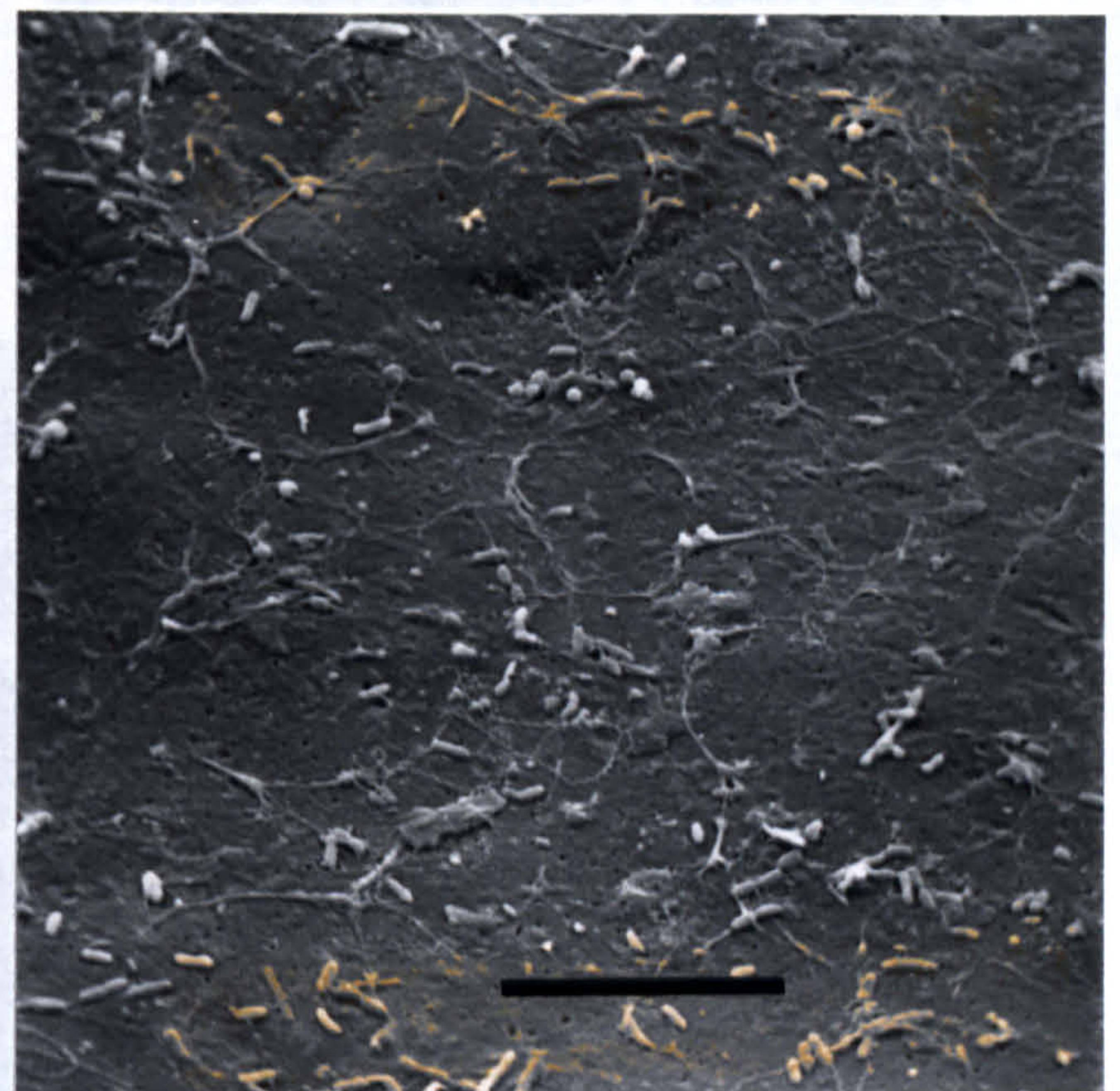
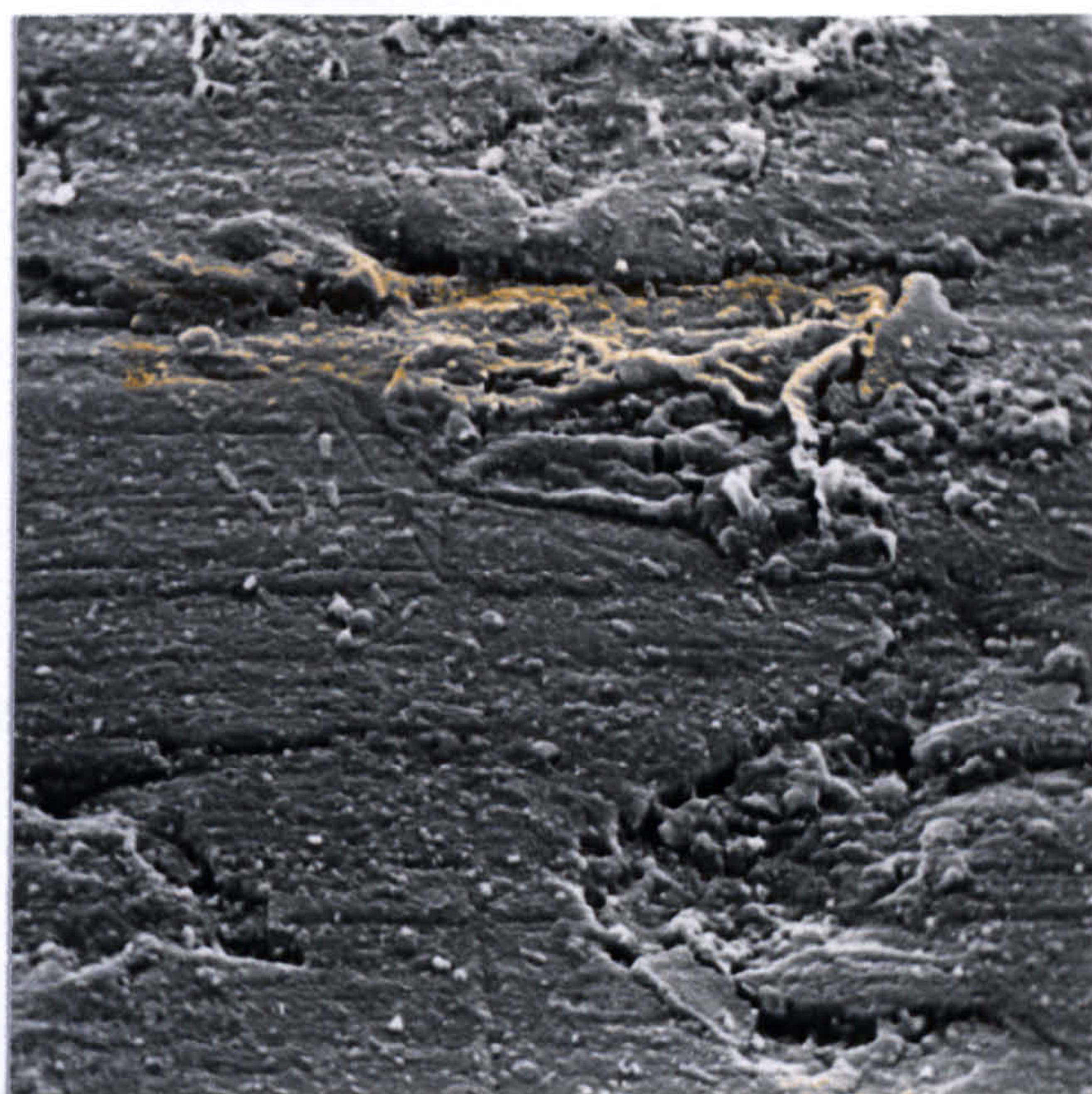
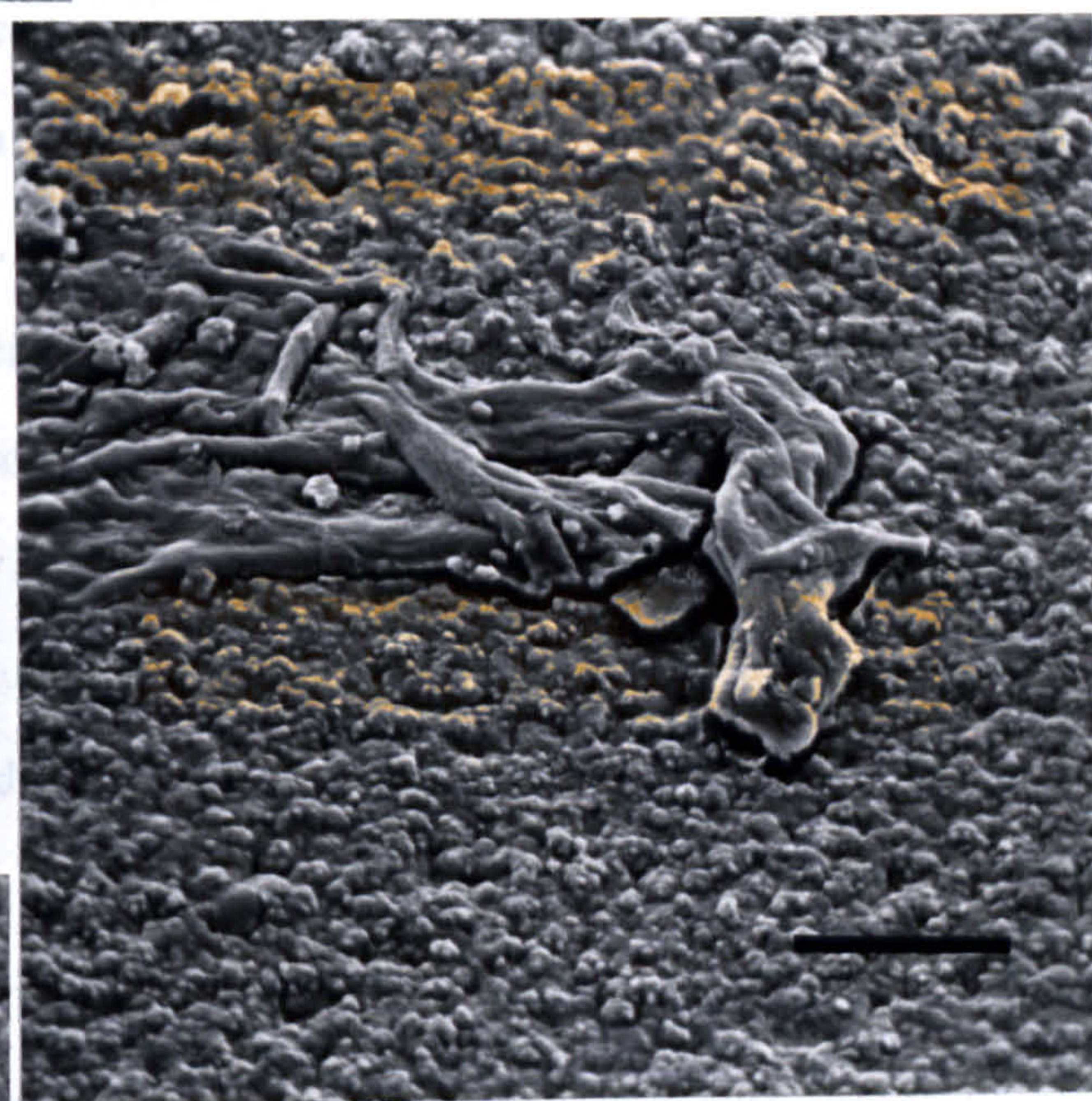


Figure 5.11 Scanning electron micrographs of biofilm formation on the surface of copper after 1 (a), 4 (b) and 21 (c) days at 40°C.



(a)



(b)



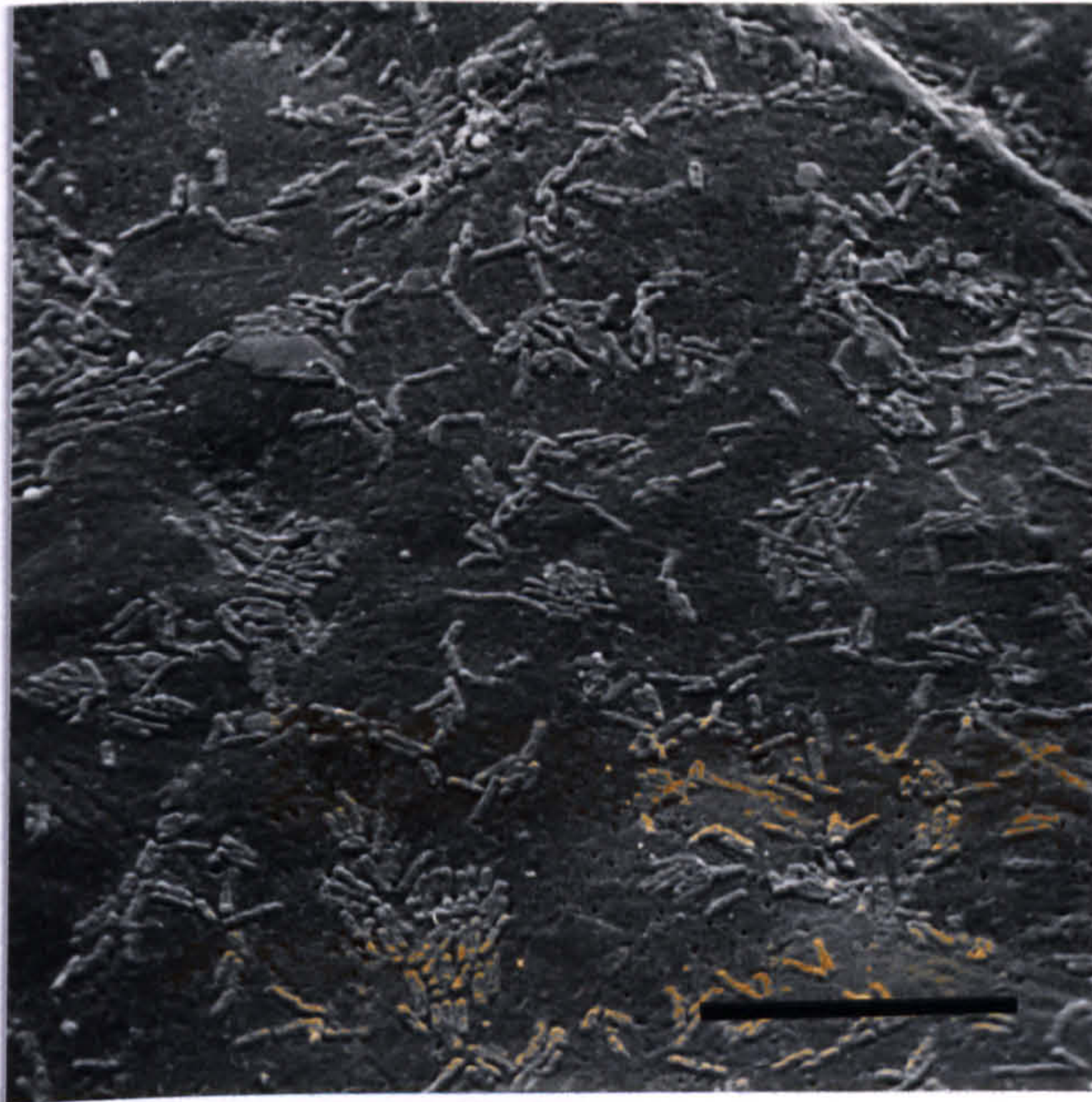
(c)

At 40°C copper surfaces were largely uncolonised by bacterial flora when viewed under the electron microscope, however, small isolated areas of the surface were able to support the growth of bacterial cells. After 24 h in the chemostat model a few bacteria and a small region of polymer could be observed on the copper surface (Figure 5.11a). The similar regions of this polymeric material could also be observed on the 4 day old copper tile (Figure 5.11b). Bacterial imprints of bacteria could be observed in the matrix on the 7 day copper tile and polymer was also evident after 21 days (Figure 5.11c). In all cases the majority of the coupon of copper appeared to have little or no bacterial colonisation.

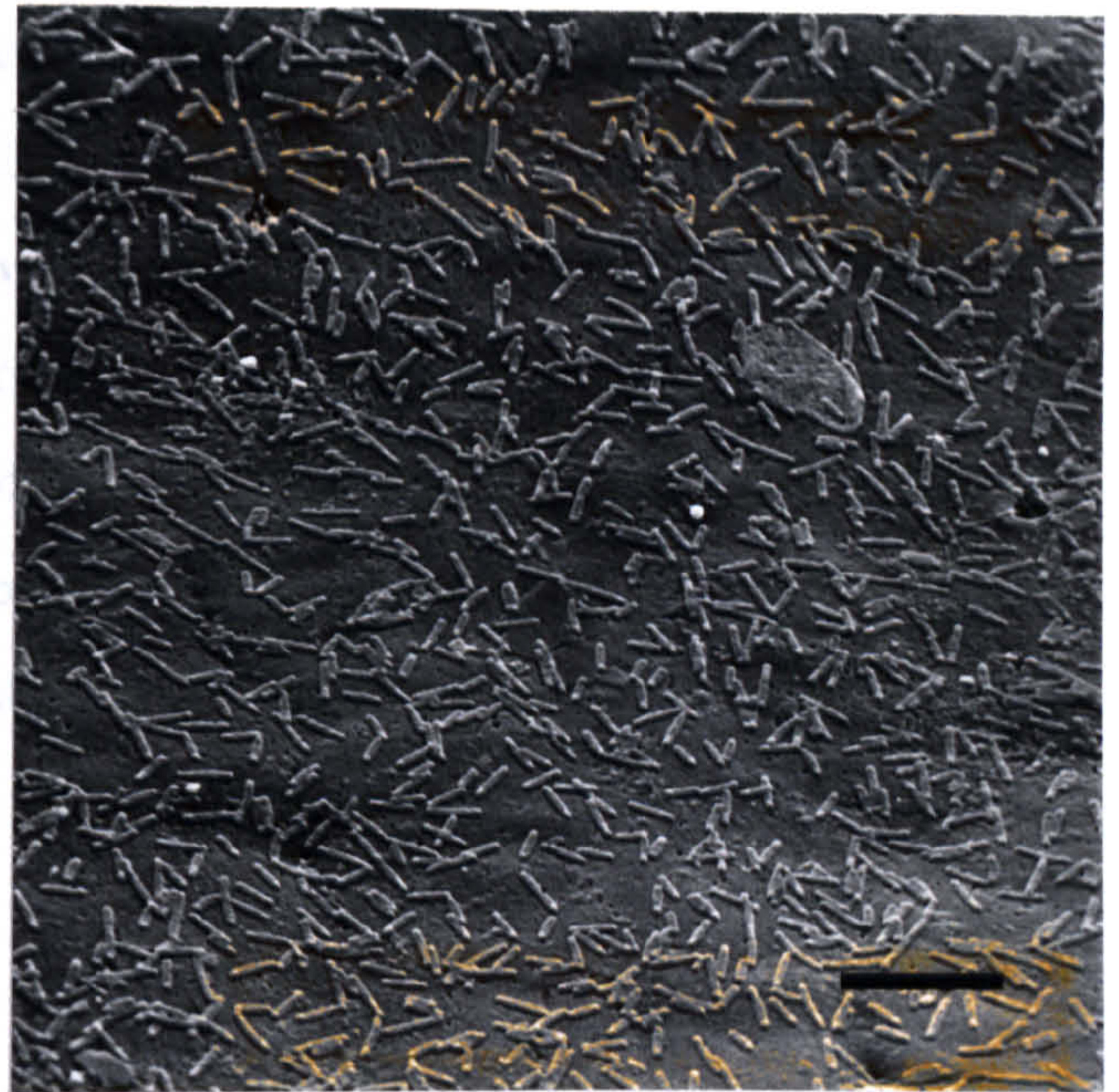
At 50°C: After 24 h the polybutylene surface supported a layer of bacteria dispersed widely over the tile in small microcolonies. The bacteria were all of similar morphology, being rods of moderate length. Occasional clusters of microorganisms had polymer associated with the bacterial cells. After 4 days the bacteria were more thickly dispersed over the surface but showed the same lack of diversity. Most cells had no extracellular material associated with them, but some had small amounts surrounding them in localised areas. As the surfaces were incubated for longer periods the calcium carbonate from the water formed a layer in which bacterial cells became embedded. After 14 days the same moderate length rods could be observed partially covered in a thick layer of calcium carbonate which covered the whole plastic surface. By 21 days no bacterial cells could be observed because the calcium carbonate layers were so extensive. The scale formed large crystals and the layer of scale had cracked exposing the depth which had accumulated.

Figure 5.12 Scanning electron micrographs of biofilms developed on polybutylene after 1 (a), 4 (b) and 14 (c) days at 50°C.

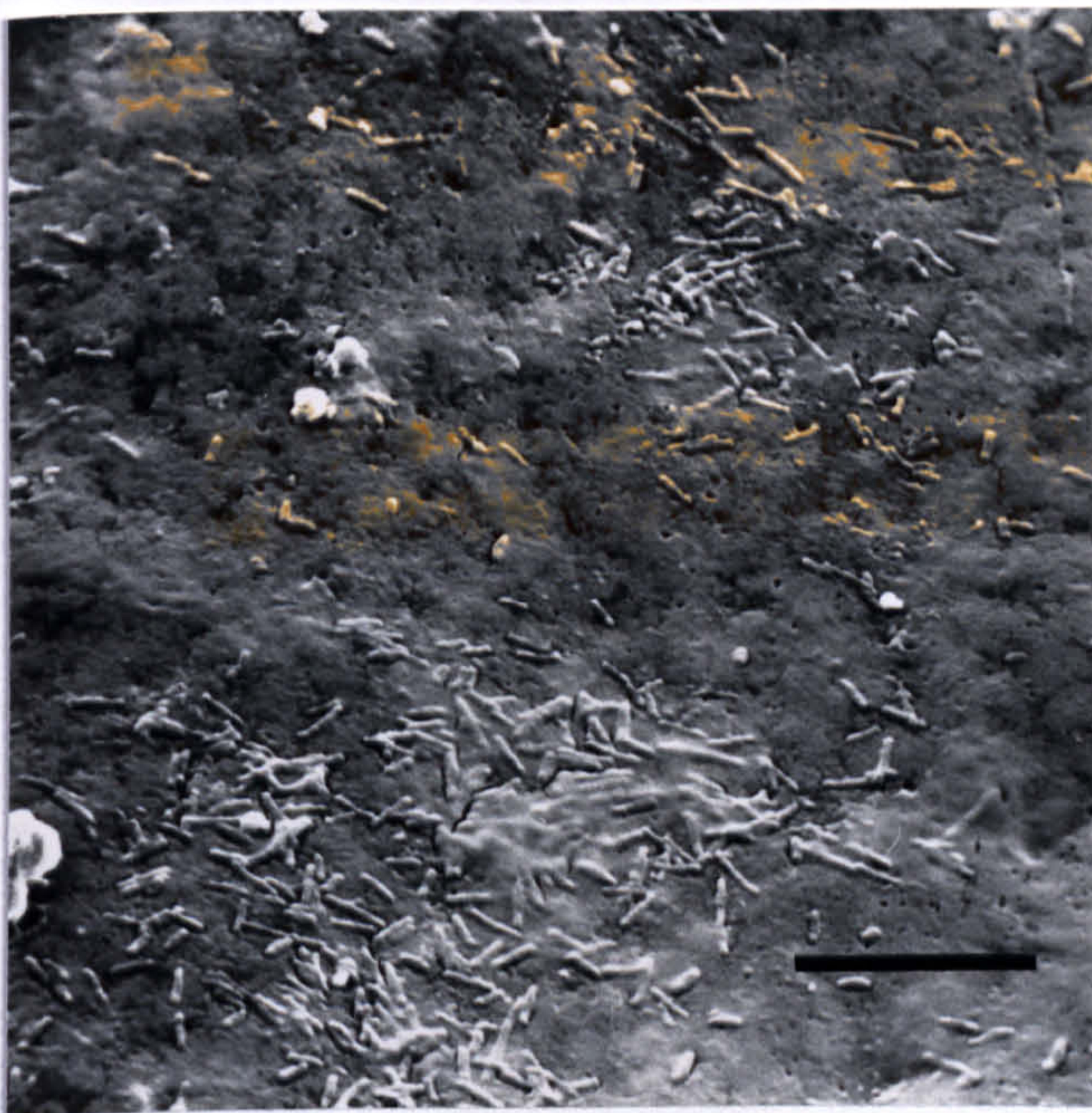
(a)



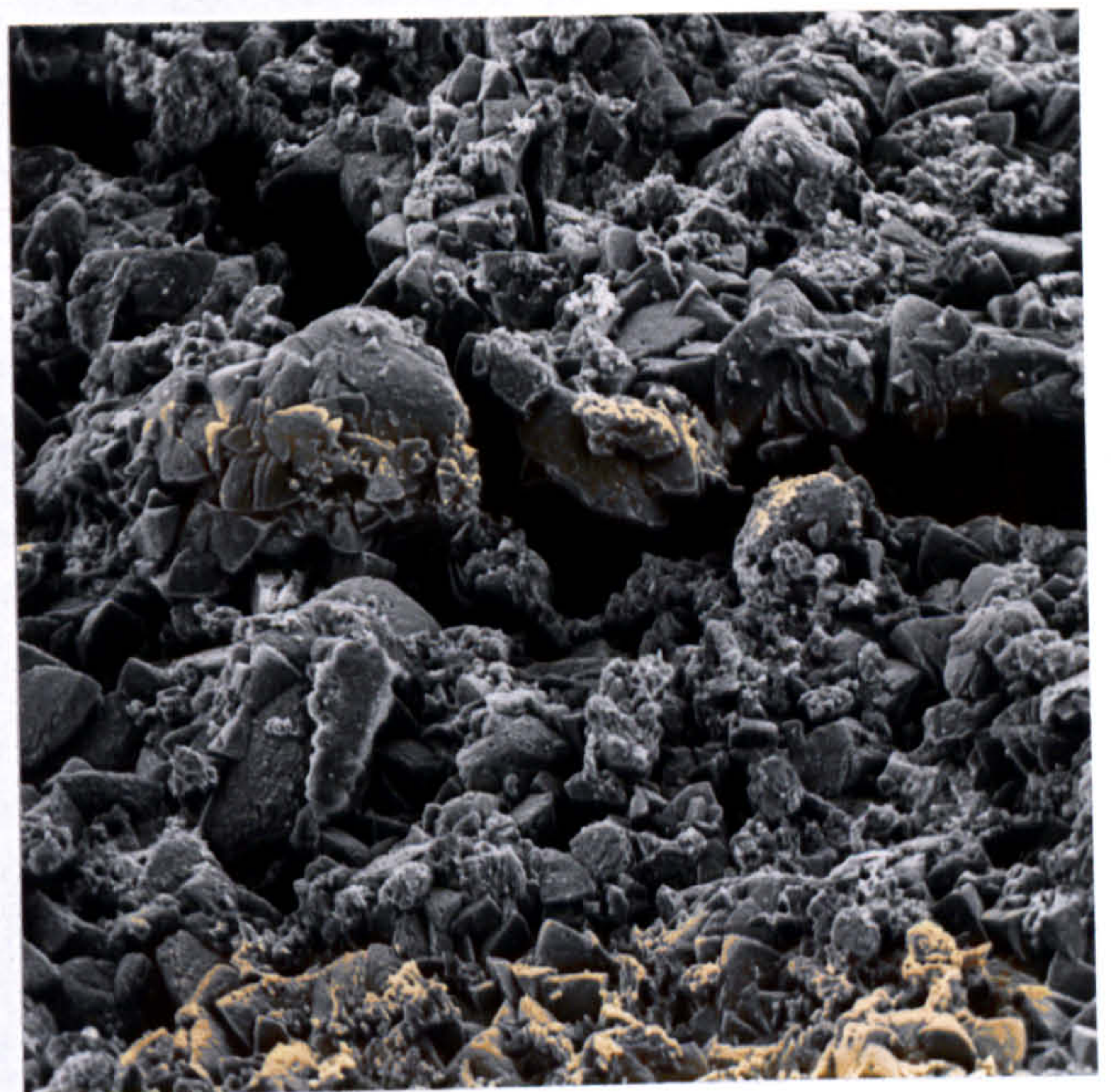
(b)



(c)

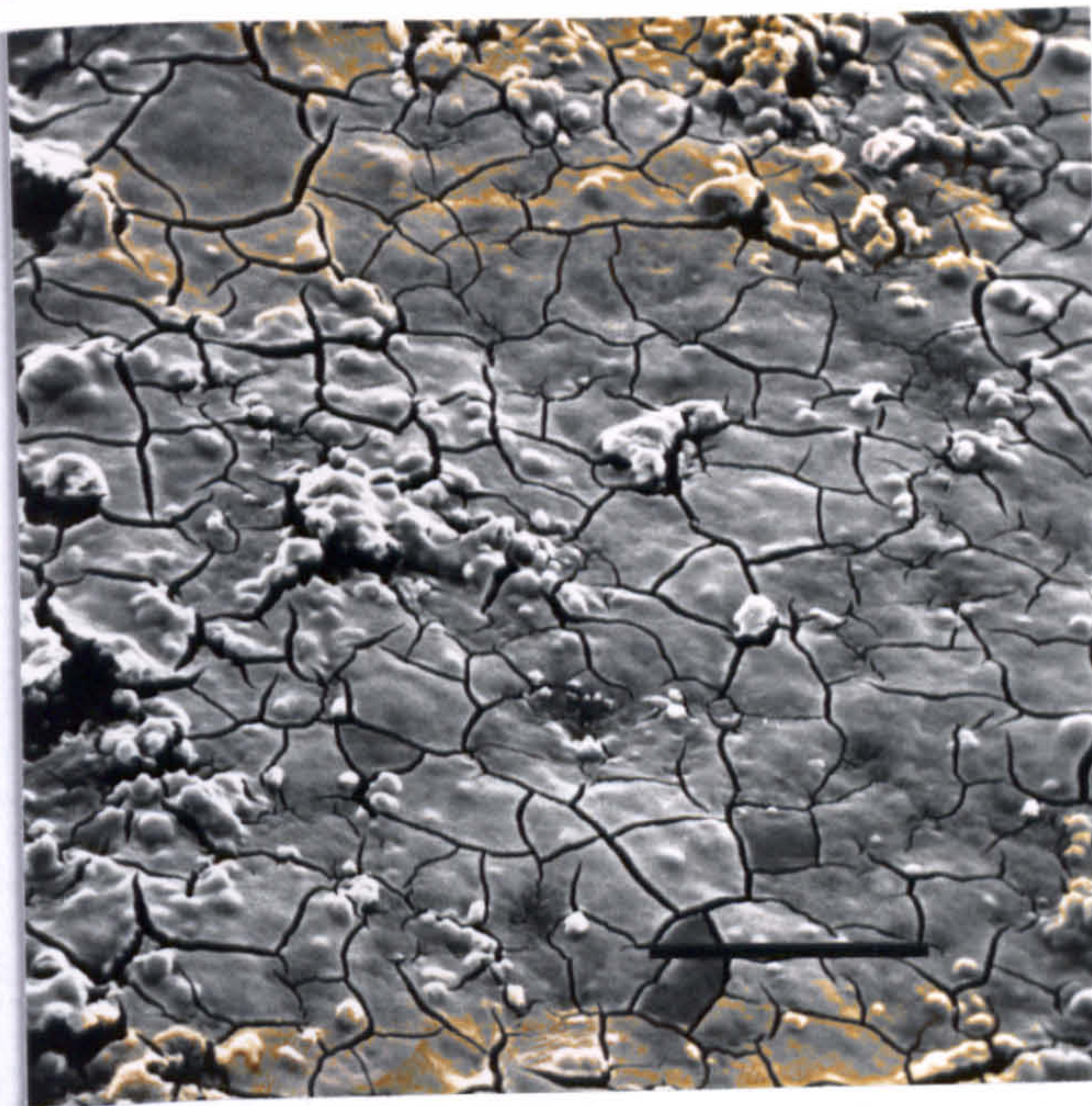


(d)

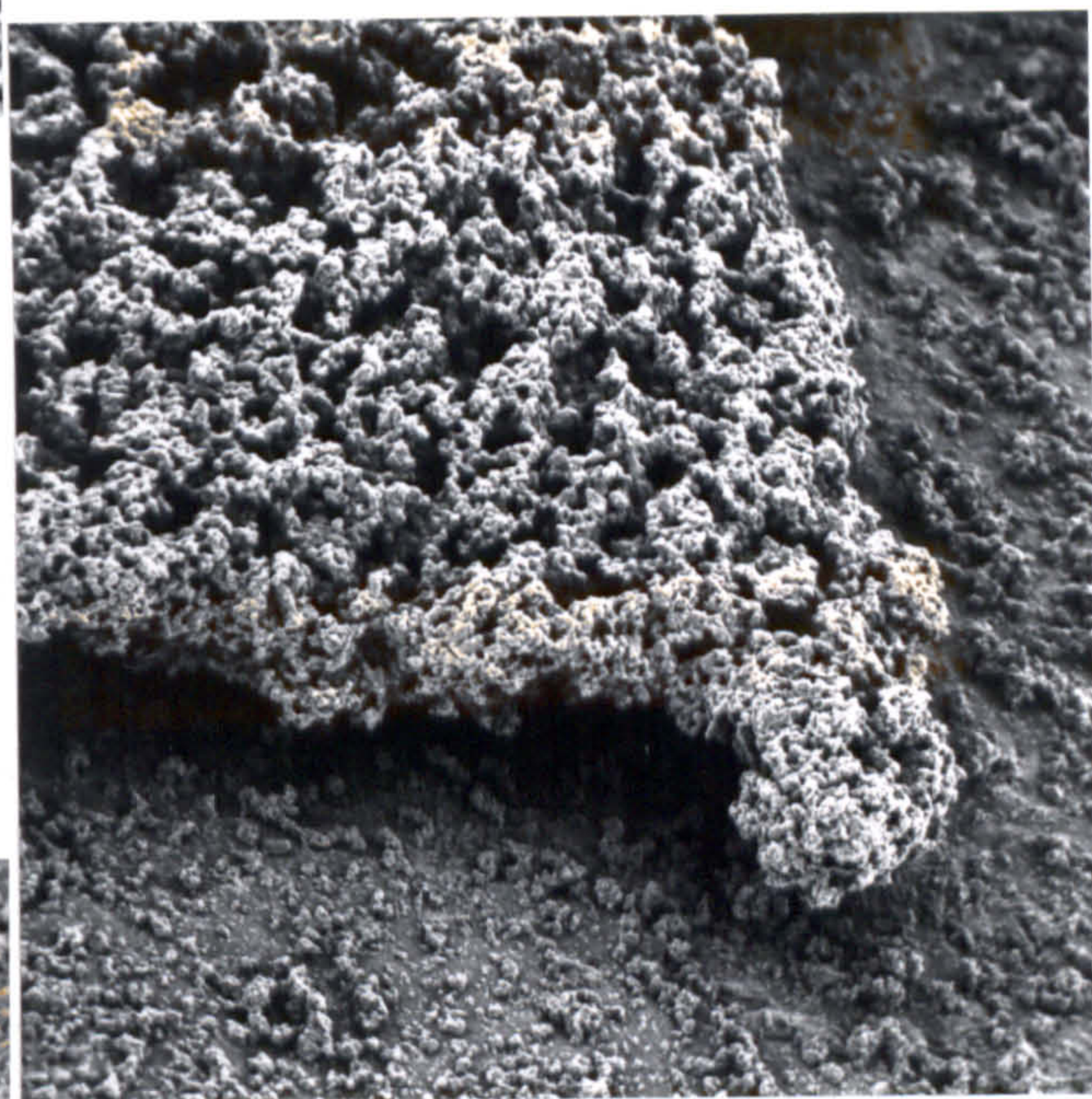


At 60°C: In all cases the biofilm developing on the various material surfaces was predominately composed of depositions of chalk from the hard water. After 24 h the glass surfaces were completely coated with a 1mm thick layer of calcium carbonate that could be easily observed by eye. As a consequence of this large amount of inert material bacterial cells could not be distinguished on any of the material surfaces. After 7 days the calcium deposits could be observed to cover the whole of the polybutylene surface. The fine crystal structure could be observed to form a confluent layer that have cracked due to the dessication procedures of sample preparation (Figure 5.13a). After 14 days larger areas of dense crystal were observed to protrude from the lower layers of calcium deposits (Figure 5.13b). The crystals present on the 21 day surface were larger and have a needle shape. These completely covered the surface and made observation of bacterial cells impossible (Figure 5.13c).

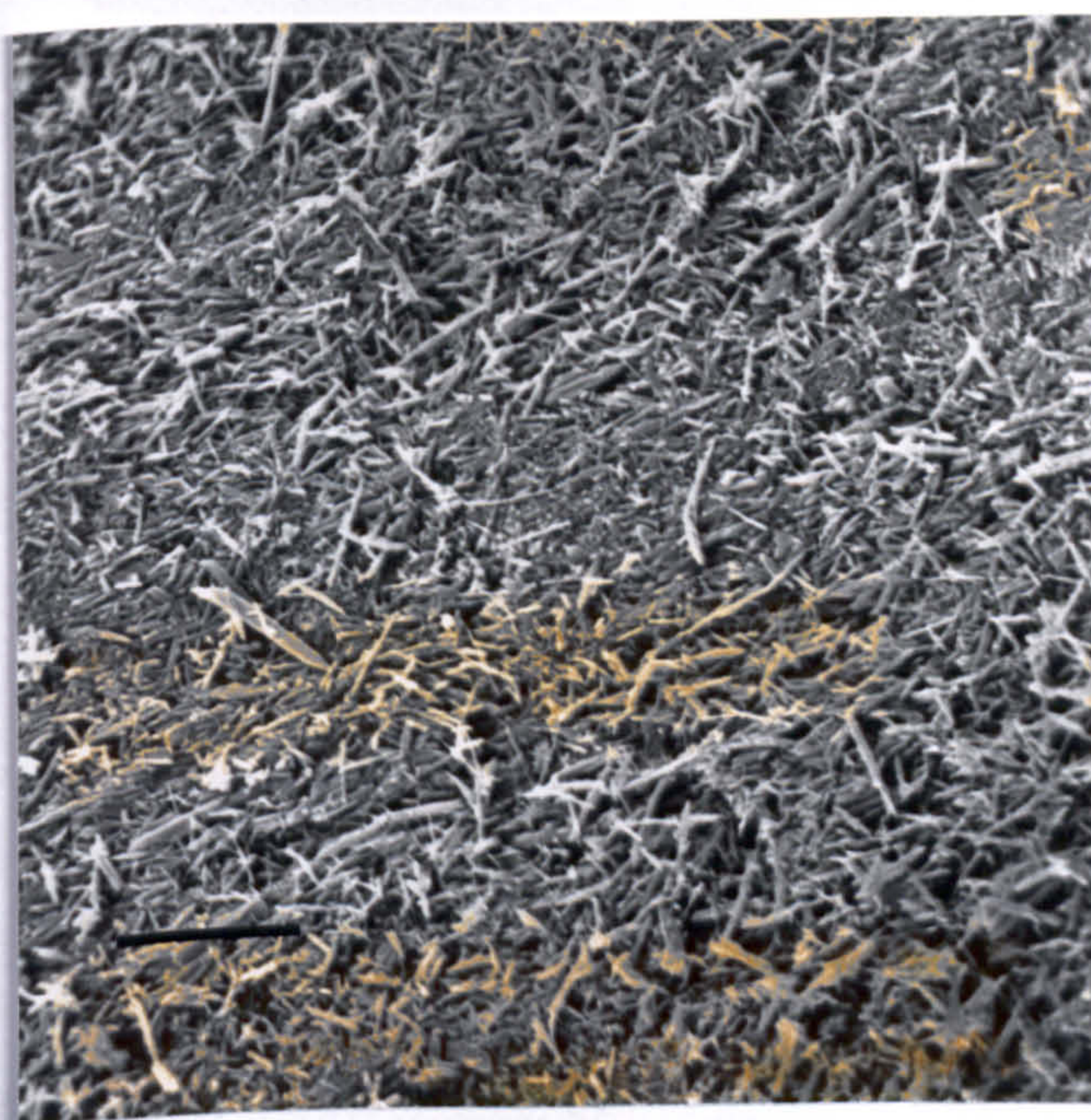
Figure 5.13 Scanning electron micrographs of biofilms developed on polybutylene after 1 (a), 4 (b) and 14 (c) days at 60 °C.



(a)



(b)



(c)

5.3.7 Leaching of copper ions into the aqueous phase and accumulation in biofilms on glass surfaces.

Samples from the aqueous phase of the model system were found to contain negligible levels of copper since, the copper concentration was below the limit of detection for the duration of the experimental phase. The detection limit was 1 mg/l.

The biofilm developed on the glass tile was found to contain 57 µg total copper (on a surface area of 2.2cm). This would suggest that although the planktonic phase contained undetectable levels of copper ions, there was leaching of copper from the surface and this copper could coat other materials and so inhibit biofilm formation on their surfaces. The biofilm was dissolved in only 1.0ml of acid and this explains why there is an apparent increase in sensitivity compared with the planktonic samples.

5.3.8 Polymer production at different temperatures

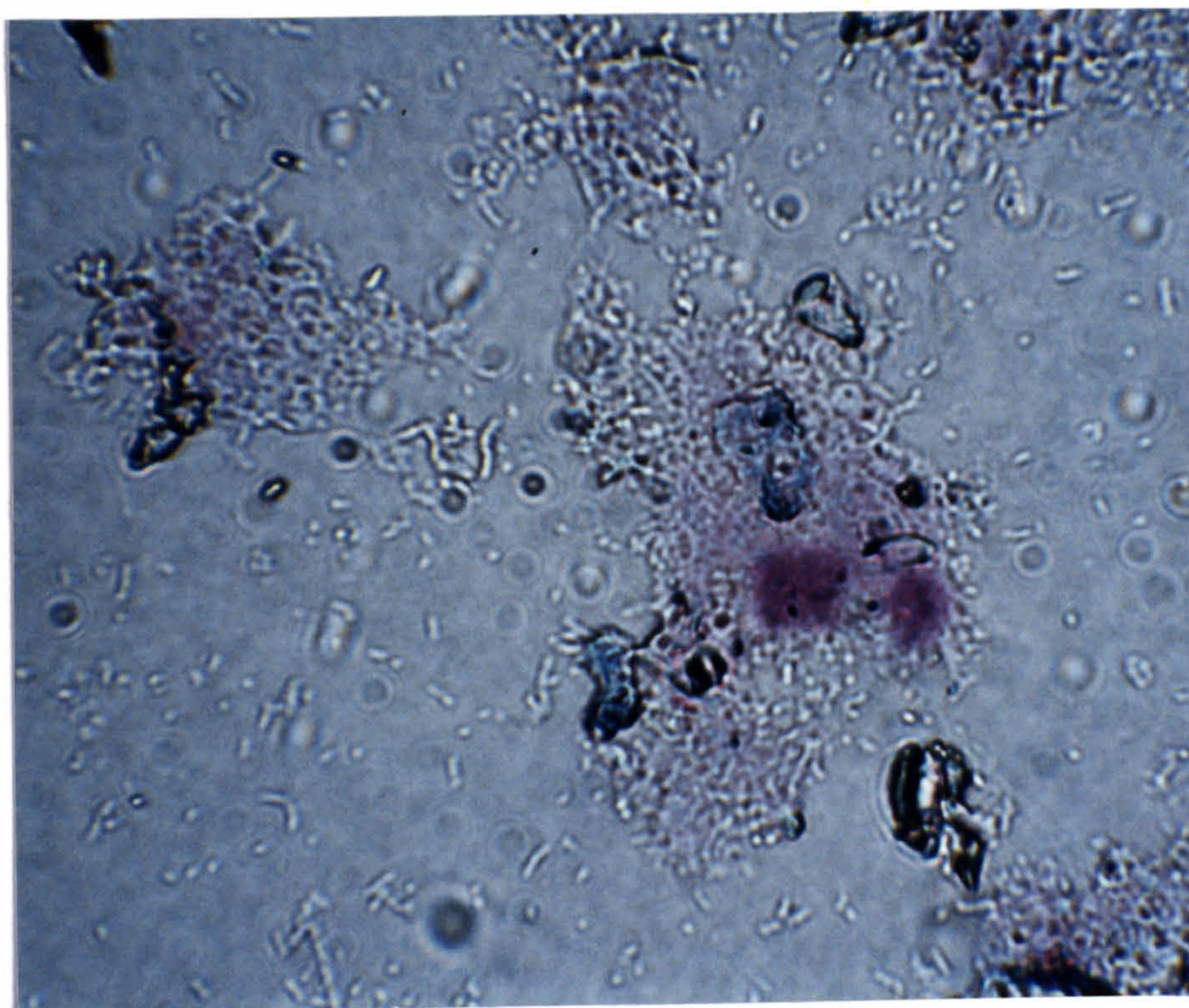
The use of the combined PAS alcian blue staining procedure gave an insight into the differing activities within the biofilm. Cells which occurred in microcolonies appeared to produce a localised amount of polymer. PAS positive polymer stains magenta using this technique because it is neutral, containing hexosamine and hexose units which do not have free acidic groups. Acidic polymers are PAS negative and stain with Alcian blue, giving an overall blue colouration. These acidic mucins consist of hexosamine units and possibly glucuric, iduronic or sialic acid, (Cook, 1972). Either sulphate radicles are present, or the reactive portion is provided by the carboxyl groups of the acids.

At 20 °C the biofilm was principally composed of regions of magenta stained polymer. There were isolated areas of the biofilm which had the blue coloured polymer present. The rotifers have alimentary canals which contain both mucin types and therefore act as controls for the staining procedure.

At 40 °C both of the polymer types were in approximately equal abundance, again they occurred in tight zones. The existence of these zones would suggest that each zone was produced by a particular species, again giving credence to the existence of microcolonies within the biofilm. At 50 °C only the blue stained polymer was evident and in sparse amounts. At 60 °C there was no evidence of polymer production.

Figure 5.14 PAS alcian blue stained biofilms developed at 20°C showing differently stained biofilm polymer (a) and rotifers (b).

(a)



(b)

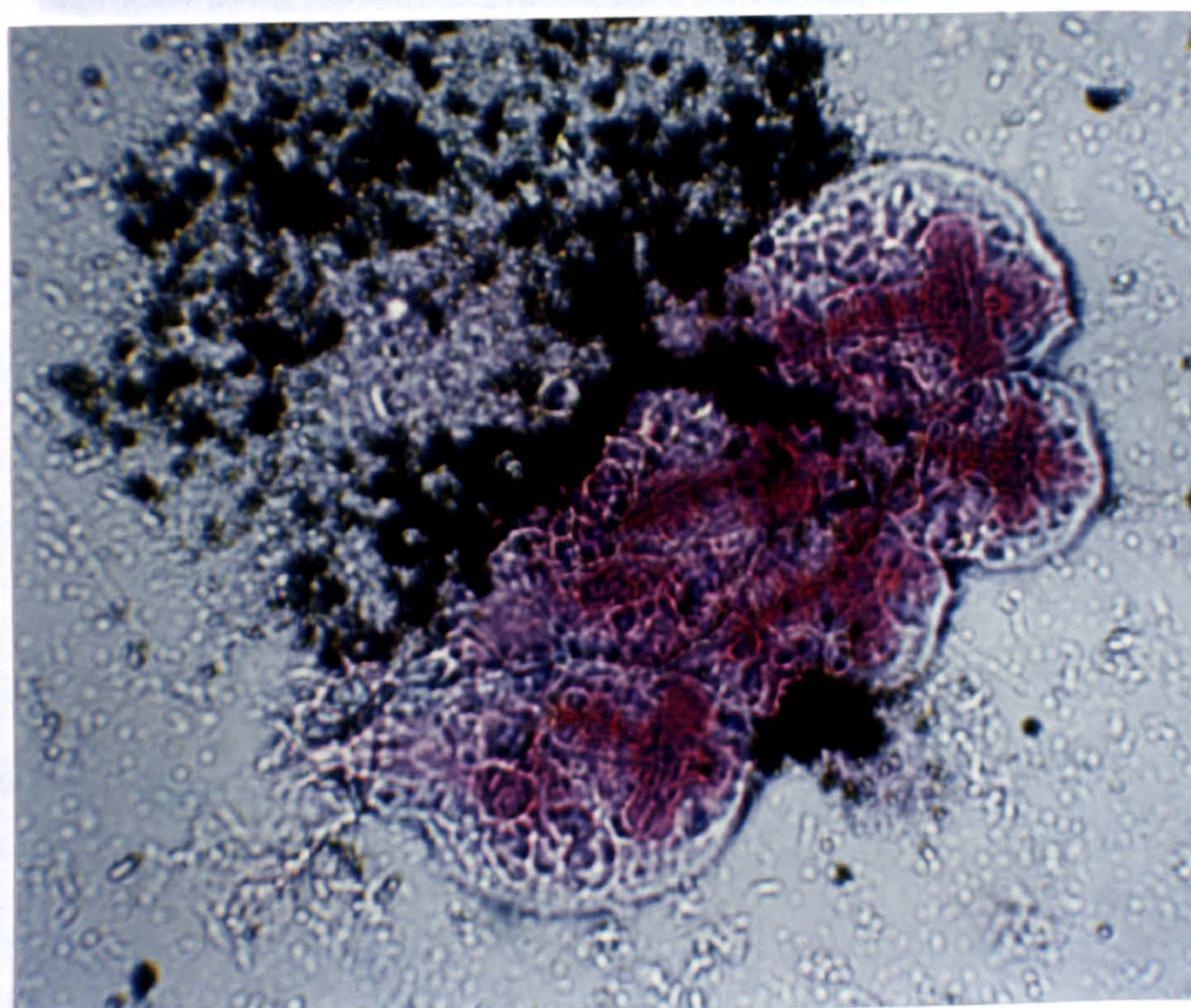
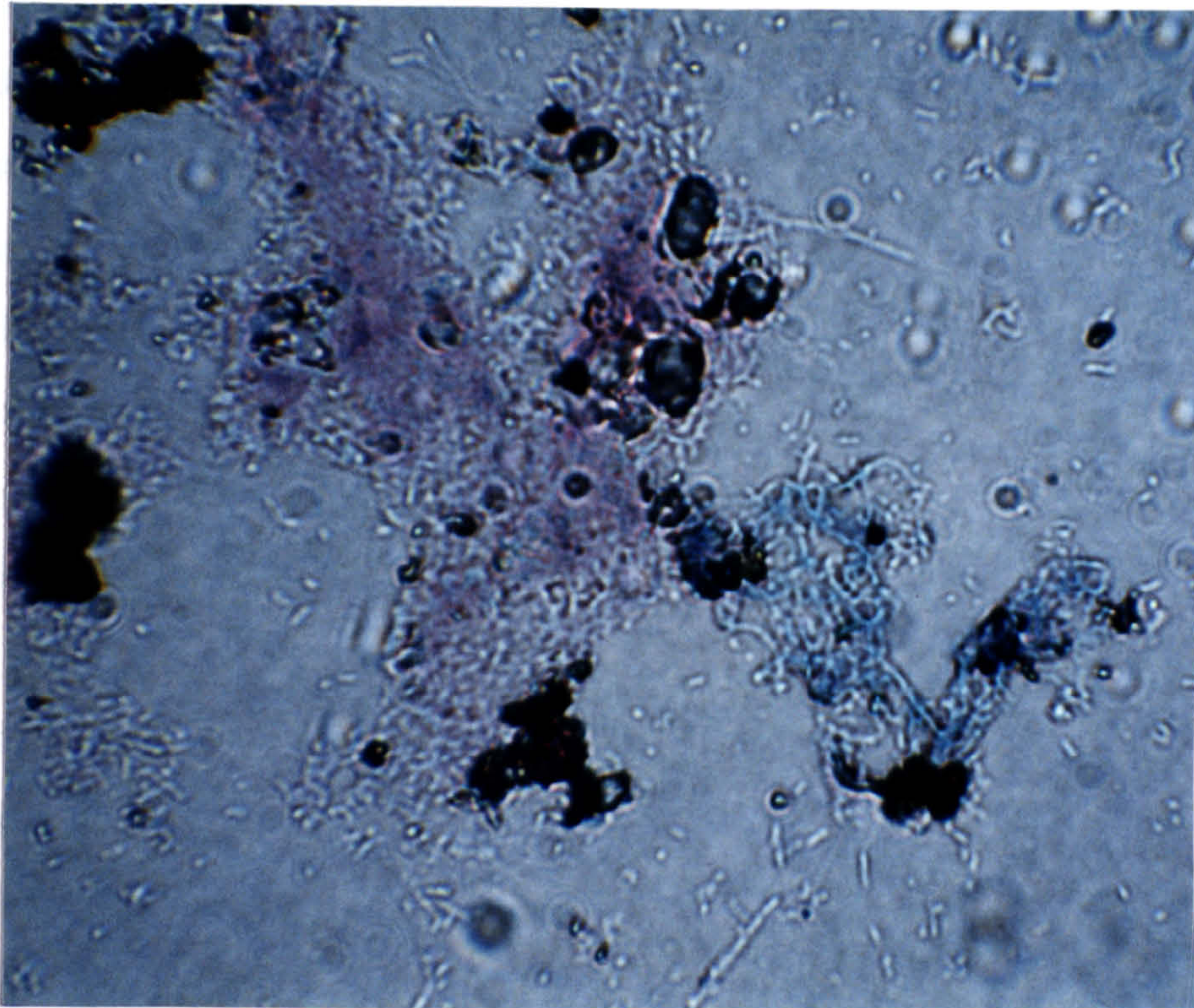
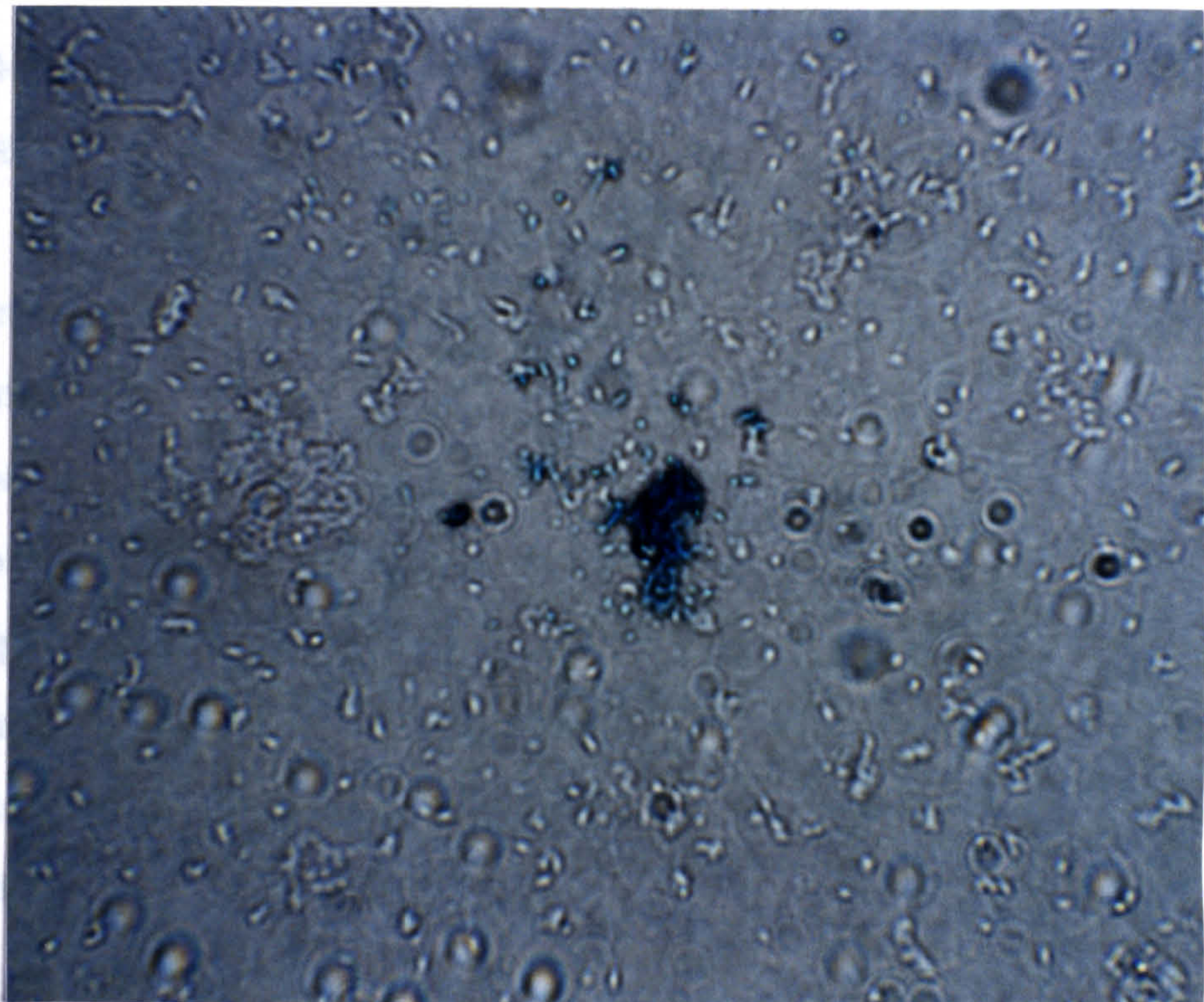


Figure 5.15 PAS alcian blue stained biofilms developed in the model system at 40°C (a) and 50°C (b).

(a)



(b)



5.4 DISCUSSION

The ability of *L. pneumophila* to grow and survive over a wide range of temperatures has been demonstrated. The *L. pneumophila* is incorporated into both the planktonic and biofilm phases of the model system at 20, 40 and 50 °C. At 60 °C the pathogen was absent from the model system. The overall trend of growth was temperature related. At 20 °C biofilms and planktonic phases contained low numbers of *L. pneumophila*. Since there were many potential hosts present in the culture and the bacterial species were diverse at 20 °C the low numbers of *L. pneumophila* within the system were probably due to their low metabolic rate at this temperature.

At 40 °C the *L. pneumophila* were at their most abundant. On the plastic surfaces half of the total population were *L. pneumophila*. The high numbers of *L. pneumophila* coincided with undetectable numbers of potential hosts. This data suggested that the bacterial consortium was amplifying the numbers of *L. pneumophila* at this temperature. Many bacteria and cyanobacterial species have been shown to be able to support growth of the pathogen extracellularly (Wadowsky *et al.*, 1985 and Tison *et al.*, 1980) and microcolonies of *L. pneumophila* within biofilms (Rogers *et al.*, 1992) would support the hypothesis that biofilm consortia could be encouraging growth of the pathogen.

When temperatures were increased to 50 °C the bacterial diversity was greatly reduced and the numbers of *L. pneumophila* within the system declined. The plastic surfaces supported numbers of *L. pneumophila* similar to those found at 20 °C. The interesting feature of these biofilms was the gradual increase in the population of the pathogen. It would appear unlikely that the *L. pneumophila* could grow at 50 °C (Dennis *et al.*, 1984) and their persistence was probably due to inflow from the first chemostat vessel rather than growth. However, the viability of the *L. pneumophila* was maintained over extended time periods indicating that the biofilm in some way protected them from the

high water temperatures. This data would suggest that hot water systems operating at 50 °C may contain a reservoir of viable *L. pneumophila* in biofilms. This may be of no consequence if temperatures are always maintained at 50 °C since numbers of the pathogen are low and so unlikely to cause infection. However, if some incident resulted in only a relatively small temperature drop, portions of the system may have conditions that both favour the growth and already contain a population of *L. pneumophila*. The rapid growth rate at 40 °C would indicate that high numbers could be reached in a short time period.

Copper was consistently less biofouled than either polybutylene or PVCc at all of the temperatures examined. The numbers of *L. pneumophila* was also consistently lower on the surface of the copper than the plastics at any of the temperatures. Copper ions could be inhibitory to growth of *L. pneumophila* directly and this could possibly account for the reductions in the pathogen populations when copper was present in the model. However, *L. pneumophila* cannot grow alone in sterile water, requiring the presence of additional microorganisms to support growth (West *et al.* 1989). Lack of growth of *L. pneumophila* in the presence of copper could be attributed to the inhibition of these nutrient supplying populations by the copper. Bacterial diversity was greatly reduced on copper surfaces compared with the plastics and this lack of supporting populations may have accounted for some of the inhibition by copper.

The copper surface appeared to leach low levels of copper ions into the culture which reduced planktonic populations and the accumulation of these ions on glass surfaces led to reduced colonisation. The inhibition was maintained over all the temperatures tested. The inhibition occurred with low levels of copper were present in the planktonic and biofilm phases and these were well within the permitted levels suggested by the Water Supply (Water Quality) Regulations 1989. These standards state that the maximum permitted quantity of copper in drinking water is 3 mg/l. This would suggest that the

use of copper tube in plumbing systems may not only prevent colonisation of the tube but could possibly also inhibit colonisation of minor non-copper components. The use of copper as a plumbing material may help to minimise the risk of Legionnaires' disease, particularly if plumbing systems were unable to operate at 60 °C. There are several reasons for the failure to comply with the guidelines, patients must be protected from scalding, energy costs are high or hospital systems are old and temperatures cannot be maintained at 50 °C. Under these conditions the use of copper tube would minimise the numbers of *L. pneumophila* and could possibly be used as part of a control measure.

CHAPTER 6

CHEMICAL CONTROL OF LEGIONELLA IN BIOFILMS

6.1 INTRODUCTION

Although it was demonstrated in chapter 5 that copper pipework is inhibitory to colonisation of water systems, it is not always a suitable material for use. In cooling towers, swimming pools and jacuzzis, for example, the systems require regular cleaning and require biocide treatment. Copper is expensive and is not mechanically tolerant of repeated disinfection and is therefore not used in these systems. The requirement of inhibition of colonisation may be achieved by the use of a suitable construction material which could be coated with a biocidal paint. Johnson Matthey had developed a potentially biocidal paint which incorporated silver which could be tested in the chemostat model.

Contact with silver has been used to keep water fresh for many years and is frequently used in ships to maintain good water quality within the stored water. The mode of silver inhibition is similar in action to that of copper, binding to DNA and to sulphhydryl groups, impairing cell division, also inactivating essential respiratory and metabolic enzymes (Thurman & Gerba, 1989). A paint incorporating silver may be successful in reducing total biofouling and limiting colonisation by aquatic pathogens such as *L. pneumophila*. A pilot study was undertaken to investigate a proprietary silver paint supplied by Johnson Matthey.

The DHSS Code of Practice (1988) recommends that cooling towers are cleaned four times a year. The cleaned systems are then flushed and refilled with clean water and usually disinfected with 20 ppm of free available chlorine. Chlorination is a relatively cheap and practicable method, but chlorine is corrosive. When water contains a high organic load (or chlorine demand) the free chlorine is rapidly utilised reducing its activity against the remaining bacteria, therefore free chlorine has to be measured to

ensure sufficient is present to be effective. In addition, when the pH of the water > 8.0 chlorine is ineffective since the molecule dissociates rapidly.

The most commonly used model for inactivation of microorganisms by disinfectants utilises the "Chick-Watson law" which states that $\ln (N/N_0) = -kC^n t$, where N/N_0 is the ratio of surviving organisms at time t , C is the disinfection concentration, and k and n are empirical constants. This implies that disinfection concentration and contact time, the $(C \times T)$ factor, are the two key variables determining disinfection efficacy. However, applications often assume complete and uniform mixing of microorganisms and disinfectant, ignoring that diffusion might be rate limiting and that disinfectant concentration might decrease with time.

Most data has been derived from the study of either monoculture studies or cooling towers. In monoculture studies the conditions are unlike the environment since the microorganisms did not normally occur in pure culture but in mixed consortia. In the case of *L. pneumophila*, the test conditions are unsuitable since growth of the pathogen could not occur in such an environment. Clearly, this type of test is inappropriate for studying the effect of biocides on aquatic microorganisms. The other factor that is overlooked in the methods is the the presence of microorganisms in biofilms and consequent relative resistance to biocide treatment.

During the testing of biocides in actual or model cooling towers mechanical conditions can be controlled, however, those factors that are particularly relevant to the growth of bacteria are not controllable, these include temperature, humidity and nutrient availability. The biocides must ultimately perform well in these systems but much data on the efficiency of biocide is required prior to this type of field trials. The chemostat model is useful in providing controlled and reproducibly conditions for testing efficiency and the use of a two-stage model ensures a constant supply of contaminated water which sustains a population of *L. pneumophila*. In actual towers other factors

may vary, possibly resulting in loss of *L. pneumophila*, which may then be incorrectly attributed to the successful biocide treatment. Importantly, cooling towers should not be operational when high numbers of the pathogen are present since exposure may lead to infection.

The chemostat model was used to generate biofilm and planktonic samples for subsequent biocide treatment with commercially available biocides. Dubact B^R is available in slow release tablet form and contains the active ingredient 1-bromo-3-chloro-5, 5-dimethylhydantoin. This reacts with water to produce two active ingredients, HOBr and HOCl. The compound is only slightly water-soluble (0.15% approx at 25°C) so it can be applied to industrial water systems as a slow release tablets. Bromination is an important alternative to chlorination in the water treatment industry because of the unique chemistry of bromine. The acid dissociation constant for hypobromous acid is one pKa unit higher than the constant for hypochlorous acid. Consequently hypobromous acid is significantly less dissociated at pH's between 7-10 and thus retains efficiency when hypochlorous acid is no longer effective. Brominated carbon compounds are also less persistent than the analogous chlorine compounds and since current legislation is demanding payment for chemical contamination of discharge water offers an incentive for replacing chlorination as a means of disinfection. This biocide has previously been shown to be successful in laboratory trials (Kurtz and Davies, 1988) and in cooling towers using a twice monthly application of 0.4 mg/l free halogen (Broadbent, 1993).

Another major group of biocides currently available for use in cooling towers is the quarternary ammonium compounds which include poly [oxyethylene (dimethylimio) ethylene (dimethyliminio) ethylene dichloride] and di isodecyl dimethyl ammonium chloride. These two biocides were tested in the model system and are nonoxidising biocides whose principal function is the control of algae.

6.2 MATERIALS AND METHODS

6.2.1 BIOCIDES TREATMENT OF BIOFILMS AND PLANKTONIC FLORA

6.2.1.1 Preparation of biofilms for biocide testing

Sterile mild steel coupons were immersed in the chemostat model operating at $30 \pm 0.2^\circ\text{C}$ and using Thames water as the nutrient source. Biofilm and planktonic samples were removed at 1, 4, 7, 14 and 21 days in order to assess microbial growth and processed as described in 2.2. At 21 days all other tiles were removed from the chemostat vessel and subjected to one of three biocide treatments. The biofilms were exposed to each biocide suspended in sterile water at varying concentration and for increasing contact times as described in 6.2.1.2. Equal concentrations of the biocides were used to treat planktonic samples from the chemostat.

6.2.1.2 Biocide treatment

GCO-30 with Visigard^R (100 and 200ppm)

The stock solution provided by the manufacturers contained fluorescein dye to aid in determining the presence of biocide in cooling towers. The stock solution contained 100 mg/l of the active ingredient poly [oxyethylene (dimethylimino) ethylene (dimethyliminio) ethylene dichloride]. The stock solution was diluted in sterile water to provide 300 ml of distilled water in order to determine the effect on biofilm and in 300 ml of chemostat effluent to determine the effect on the planktonic flora. Mild steel tiles supporting biofilm were placed in bottles containing the biocide diluted in sterile distilled water. The biofilms were incubated with the biocide for intervals of 0.5, 1, 4,

24, and 48 hours. After the contact period the biofilms were removed from the surface of the mild steel, diluted in 1ml of sterile distilled water and serially diluted to 10^{-4} as described in 2.2. The bacterial suspensions were plated on to R2A, BCYE and GVPC media and incubated at 30°C for seven days. Aliquots were removed from the chemostat effluent treated with the biocide simultaneously as the biofilm samples and the planktonic survivors plated onto the agar media in the same manner. The experiment was repeated using 200 mg/l of biocide on both planktonic and biofilm samples.

WCO-130-30E (with Penetrex^R type biodispersant) (300 and 600ppm)

The concentrated biocide and biodispersant were diluted in 300 ml of distilled water or 300ml of chemostat effluent to a final concentration of 300 mg/l.

Following the method used for the GCO-30 with visigard biofilm on mild steel tiles was incubated with biocide in sterile water for 0.5, 3, 5, 7, 24, and 48 hours. The tiles were then removed from the biocide and samples treated as described for Section 2.3.

The biocide efficiency was also tested using 600 gm/l of WCO-130-30E with biodispersant.

Dubact B^R

Dubact B is an oxidising bromicide supplied in solid form. The active ingredient comprised 92.5 % of the tablet and was 2-2 bromo chloro dimethylhydantoin. On dissolution 30.6 % bromine and 13.6 % chlorine was released. The tablets were crushed and dissolved in sterile distilled water. The concentration of combined bromine and chlorine was determined colorimetrically using the Lovibond Palintest DPD Tablets in a comparator against the bromine filter (The Tintometer Ltd., Salisbury, England). The concentrated bromicide was diluted to 10 mg/l in distilled water and 10 mg/l Penetrex biodispersant was added. The preformed biofilms on the mild steel tiles

were aseptically removed from the chemostat at day 21 and immersed for 1, 4, 7, 24, and 48 hours in the bromicide. The biofilm was removed using a sterile dental probe, serially diluted and plating onto GVPC and R2A media to recover bacterial survivors. No neutralising agents were used (see Section 6.4). Samples from the planktonic phase were treated with biocide at the same concentration as the biofilms. Samples were then processed as described in 2.3.

6.2.2 The evaluation of antifouling properties of a silver containing paint

The model system was used for the evaluation of biofilm development on glass tiles sprayed with a proprietary silver paint, developed by Johnson Matthey for use in cooling towers or swimming pools. Biofouling of this surface was compared with colonisation of glass with and without the presence of the silver containing paint.

The equipment was set up as described in Section 2.1 using Thames water as the nutrient source and the materials were evaluated in the third vessel at 30 ± 0.2 °C. Sterilisation of the silver containing paint was achieved using gamma irradiation to avoid excessive heating of the paint.

Tiles were all added to the third chemostat vessel at the start of the experiment and then a glass control and silver painted tile was removed from the vessel after 1, 4, 7, 14, 21 and 28 days. Glass tiles were also inserted to the second vessel at the onset of the experiment and these were removed simultaneously from the vessel to act as glass controls in the absence of the silver painted tiles.

6.3 RESULTS

6.3.1 THE EFFECT OF THREE BIOCIDES ON BIOFILM AND PLANKTONIC GROWTH

6.3.1.1 Growth of micro-organisms in the planktonic phase

Prior to the introduction of the mild steel tiles into the aqueous phase of the chemostat, the total number of viable micro-organisms in the water was 4.0×10^5 cfu ml⁻¹ (Figure 6.1). Following insertion of the material bacterial numbers decreased to 1.24×10^5 cfu ml⁻¹. The numbers gradually increased to a maximum of 4.1×10^5 cfu ml⁻¹ after 14 days. The initial reduction in the total planktonic flora could be due to preferential attachment to the mild steel. The recovery in the numbers of the planktonic flora coincided with the rusting of the steel coupons which resulted in discolouration of the aqueous phase.

6.3.1.2 Growth of *L. pneumophila* in the planktonic phase

The viable counts of *L. pneumophila* in the planktonic phase exhibited a similar reduction following introduction of the mild steel. Their numbers decreased from 2.1×10^3 cfu ml⁻¹ (0.5% of the total population) to 1.6×10^2 cfu ml⁻¹ (Figure 6.1). After 21 days, the total viable counts increased above the initial count to 7.1×10^3 cfu ml⁻¹ (4.1% of the planktonic population growing in the presence of the rusting steel). Removal of the tiles caused the viable count to fall in the chemostat to 9.1×10^1 cfu ml⁻¹ for at least 7 days. The initial reduction in the numbers of *Legionella pneumophila* were again attributed to preferential attachment to mild steel.

Figure 6.1 Total numbers of *L. pneumophila* (▲) and other planktonic bacteria (●) in the presence and subsequent removal (↓) of mild steel.

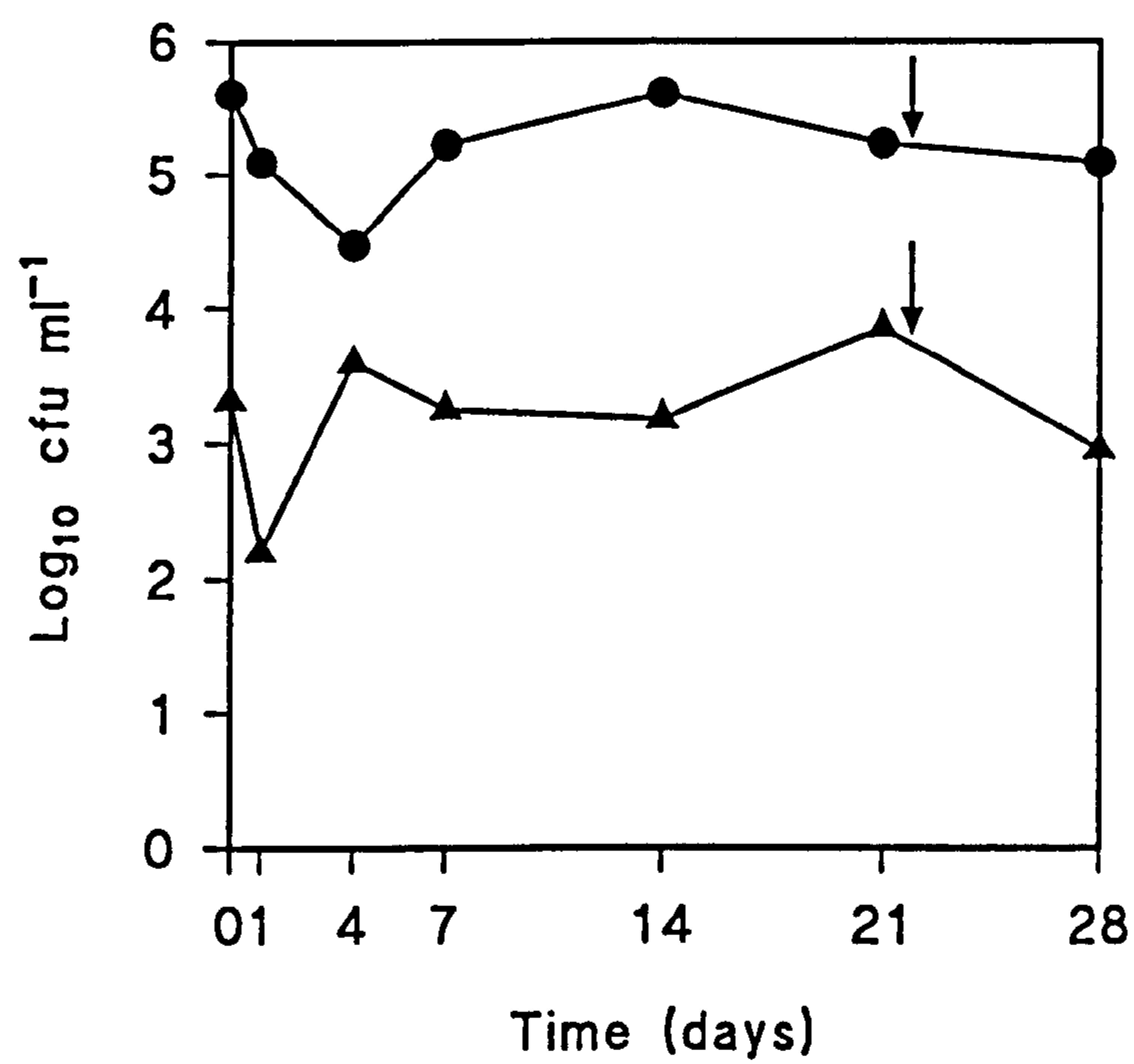
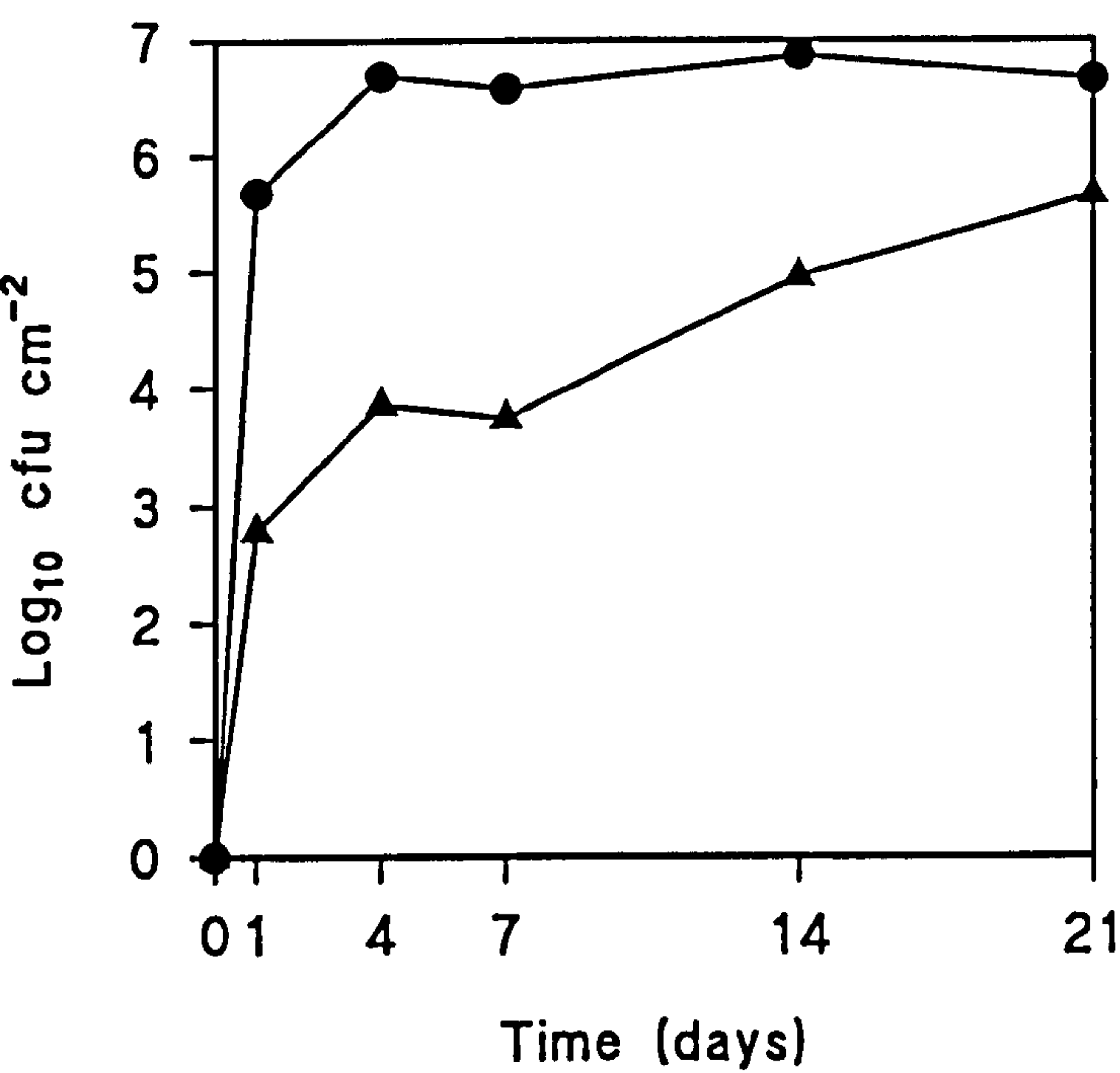


Figure 6.2 The numbers of *L. pneumophila* (▲) and other bacterial flora (●) in biofilm developing in biofilm on mild steel.



6.3.1.3 The development of biofilm on mild steel

The mild steel surface was rapidly colonised by the bacterial flora with a total count of 4.8×10^5 cfu cm⁻² after only 24 hours (Figure 6.2). Biofouling increased to 4.9×10^6 cfu cm⁻² after 4 days and fluctuated only slightly for the remainder of the experiment. By the 21st day, when biofilms were exposed to biocide, the viable count was 4.6×10^6 cfu cm⁻².

Legionella pneumophila was present in the biofilm at a concentration of 6.2×10^2 cfu cm⁻² after 24 h. Numbers increased after 4 days to 7.2×10^3 cfu cm⁻². The numbers continued to increase and by the 21st day the viable counts were 4.5×10^5 cfu cm⁻².

6.3.1.4 Population profiles of the biofilms.

During the initial phase of biofilm development the mild steel tiles were preferentially colonised by gram-negative organisms other than *Pseudomonas spp*, including *Acinetobacter*, *Alcaligenes*, *Flavobacterium*, *Methylobacterium*, and *Actinomyces spp* and *L. pneumophila* represented 0.22% of the total population. As the biofilm development proceeded, the pseudomonads became more abundant, although the other gram negative organisms still constituted a major proportion of the biofilm. The proportion of legionella in the biofilm increased with time and represented 9.8% of the total flora by the 21st day. Growth of legionella appeared to occur in the biofilm on the rusting steel surface in preference to the planktonic phase.

6.3.1.2 Determination of biocide efficacy

GCO-30 (with VisigardR)

Prior to biocide treatment the planktonic phase contained a total population of microorganisms of 1.70×10^5 cfu ml⁻¹ with legionellae at a concentration of 7.10×10^3 cfu ml⁻¹. When microorganisms from a planktonic sample were exposed to 100 mg/l of the biocide there was an initial increase of total flora and legionella (Figure 6.3). The initial enhancement of recovery was probably due to the dispersal of small clumps of microorganisms which could not be separated during vortexing. Despite a drop in the viable counts at a 1 hour contact time the microbial flora remained consistently high over the 48 hour time course. After 24 hours contact time with 100 mg/l GCO-30 (with Visigard) the numbers of both total flora and legionella were similar to the onset of the biocide treatment, implying the biocide was ineffective at this concentration (Table 6.1).

The relative resistance of the planktonic flora to the biocide was also found to be true for the biofilm results (Figure 6.4). Biofilms suspended in sterile water containing 100 mg/l GCO-30 (with Visigard) was found to reduce total biofouling after 1 h exposure by 10 %. The biocide was then ineffective in further reducing the biofilm flora. After 24 h there was 1.44×10^5 cfu cm⁻² of total biofilm flora remaining, with 1.00×10^4 cfu cm⁻² of legionella being present.

Figure 6.3 Effect of 100 mg/l GCO-30 (with Visigard) on total bacterial flora (●) and *L. pneumophila* (▲) in the planktonic phase

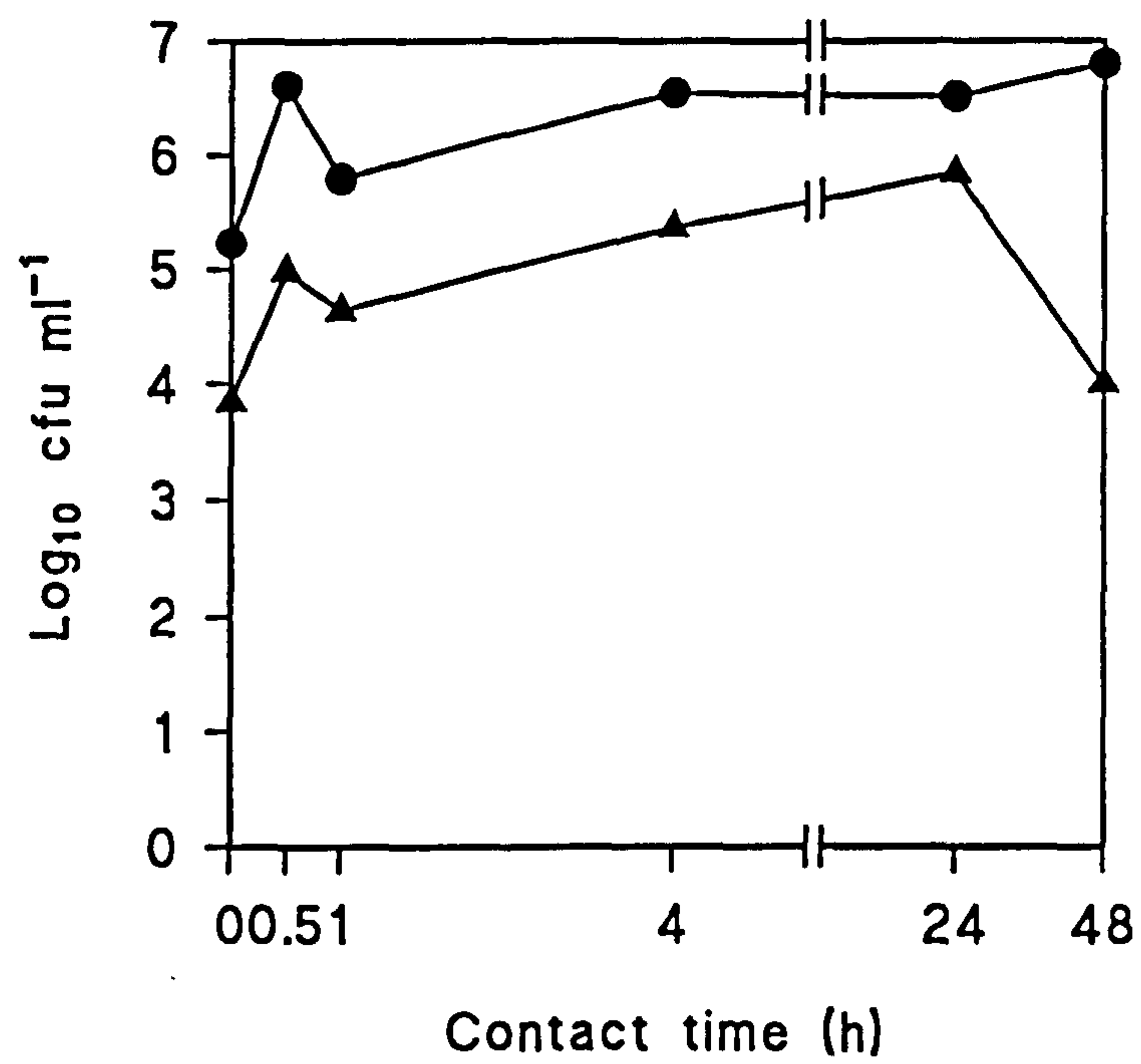


Figure 6.4 Effect of 100 mg/l GCO-30 (with Visigard) on total bacterial flora (●) and *L. pneumophila* (▲) in biofilms developed on mild steel

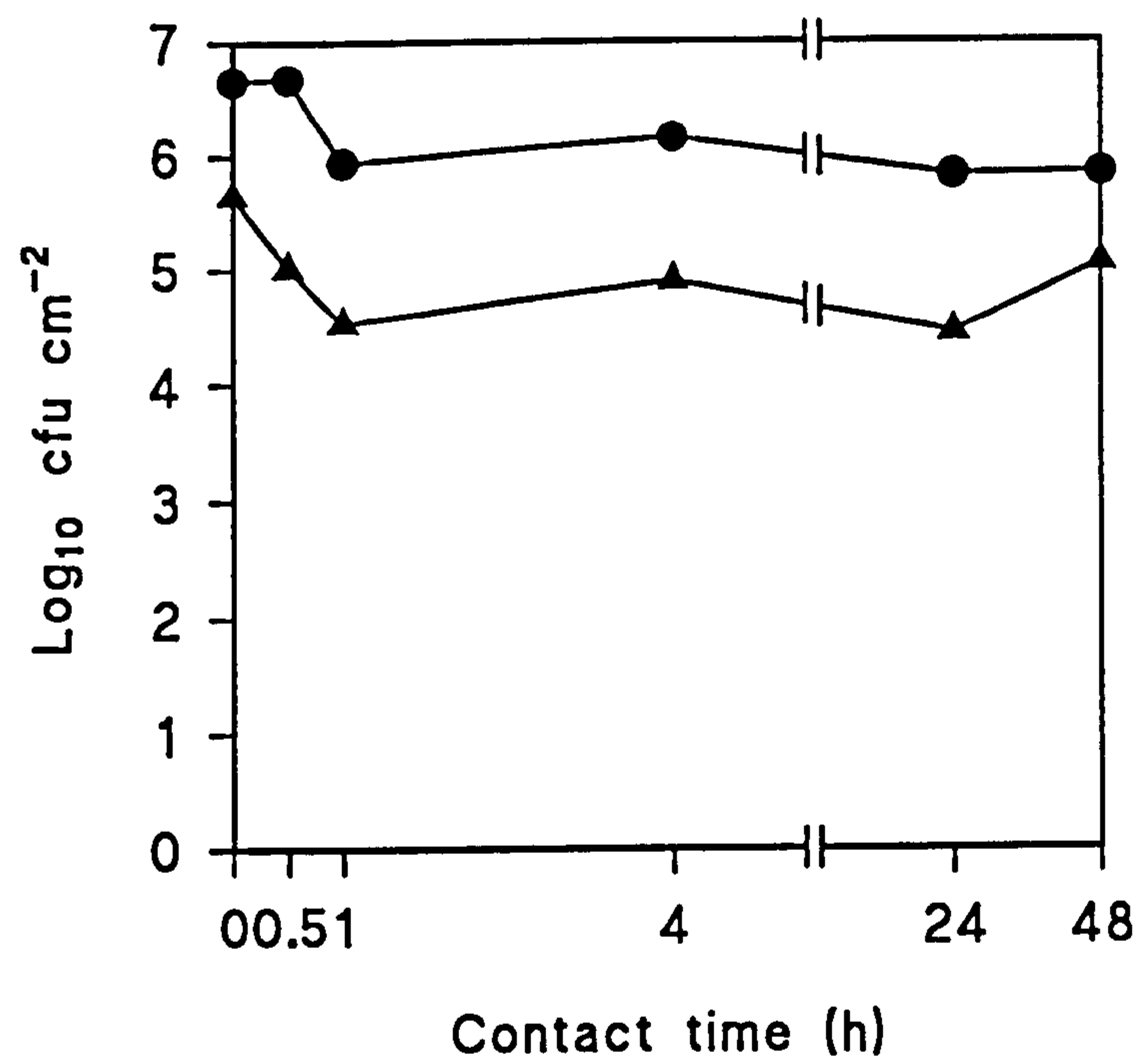


TABLE 6.1 Resistance to biocide of planktonic and biofilm populations containing *L. pneumophila* on mild steel (ND denotes not done)

Biocide	Planktonic (cfu ml ⁻¹)		Biofilm (cfu cm ⁻²)	
	non-legionella	<i>L. pneumophila</i>	non-legionella	<i>L. pneumophila</i>
control	2.0x10 ⁵	2.0x10 ³	5.0x10 ⁶	5.0x10 ⁵
GCO-30				
100 mg/l	3.3x10 ⁶	6.9x10 ⁴	1.4x10 ⁵	1.0x10 ⁴
200 mg/l	2.9x10 ⁶	3.8x10 ⁴	5.0x10 ⁵	5.0x10 ⁴
400 mg/l	2.6x10 ³	2.1x10 ²	ND	ND
WCO-130-30E				
300 mg/l	<10	<10	1.3x10 ⁵	5.0x10 ²
600 mg/l	<10	10	6.8x10 ⁴	<10
Dubact B				
10 mg/l	<10	<10	4.0x10 ⁴	1.0x10 ²

The limit of detection was 10 cfu cm⁻² or 10 cfu ml⁻¹. All samples were exposed to GCO-30 for 24h, WCO-130-30E for 48h and Dubact B for 30h.

Following the poor biocidal activity of GCO-30 at 100 mg/l despite prolonged contact times, the concentration was increased to 200 mg/l. In the planktonic phase, the biocide had a similar effect at 200 mg/l as at 100 mg/l (Figure 6.5). The total numbers of microorganisms increased to a maximum of 8.90×10^6 cfu ml⁻¹ after 4 days contact time, again indicating that the biocide served only to disperse clumps of bacteria. The numbers of legionella increased to a maximum of 1.86×10^5 cfu ml⁻¹ after 30 min exposure.

Biofilm total flora and legionella were reduced by around 10 % after 1 h exposure to 200 mg/l GCO-30 (with Visigard) in sterile water (Figure 6.6). The biofilm remained stable for the remainder of the contact time with a concentration of 5.00×10^5 cfu cm⁻² total flora and 5.00×10^4 cfu cm⁻² legionella.

Following the inability of the biocide to significantly affect the total population of micro-organisms and legionella at its recommended concentrations of 100 or 200 ppm, the dose of 200 ppm was doubled to 400 ppm. The planktonic phase was investigated as no remaining biofilms were available for further testing. The numbers of viable bacteria recovered decreased markedly to approximately 10% of the original values (Figure 6.7). After 24 hours contact time the total population in the planktonic phase was 2.06×10^3 cfu ml⁻¹ and legionella were present at a concentration of 2.05×10^2 cfu cm⁻².

Figure 6.5 Effect of 200 mg/l GCO-30 (with Visigard) on total bacterial flora (●) and *L. pneumophila* (▲) in the planktonic phase.

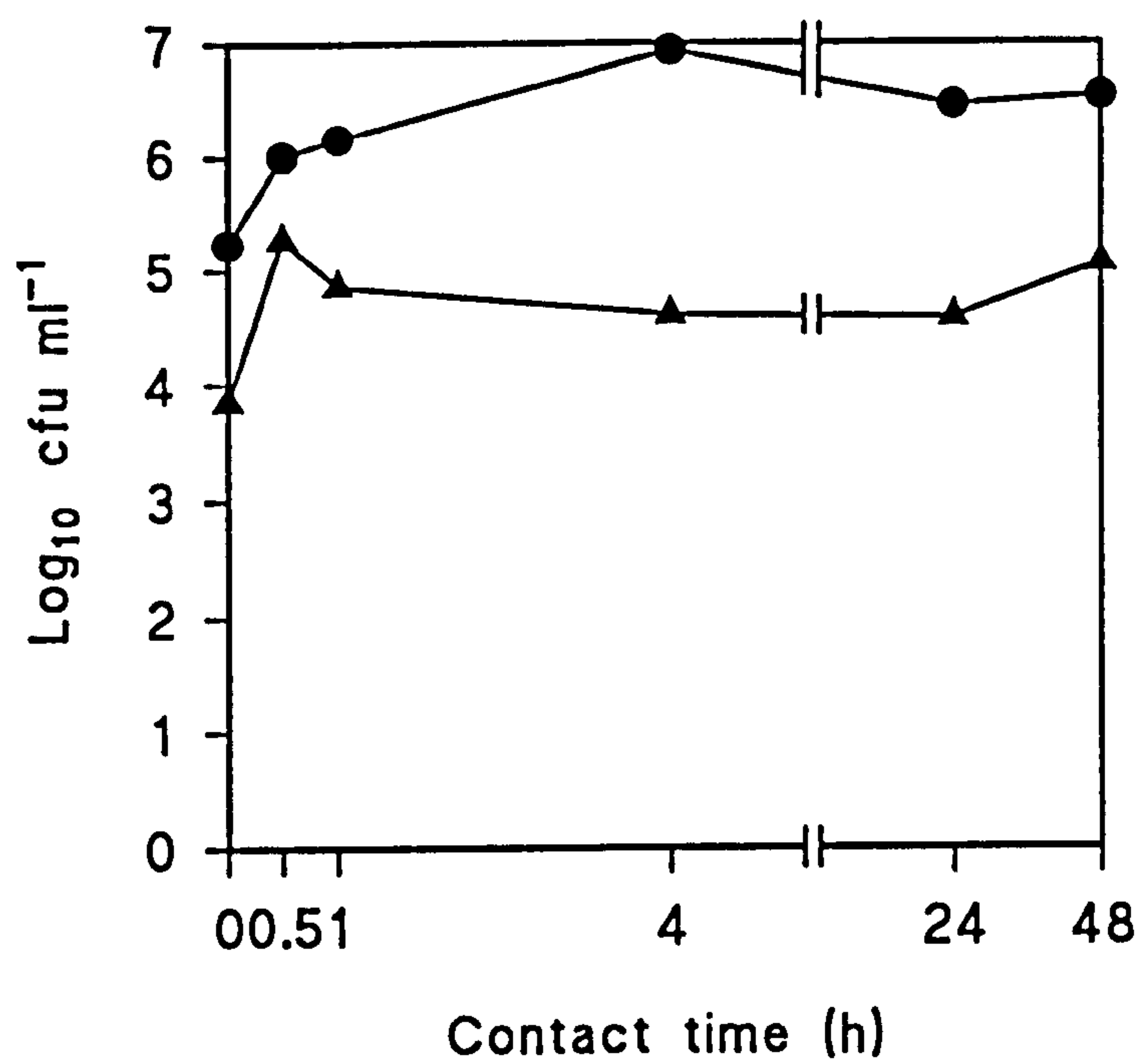


Figure 6.6 Effect of 200 mg/l GCO-30 (with Visigard) on total bacterial flora (●) and *L. pneumophila* (▲) in biofilms developed on mild steel.

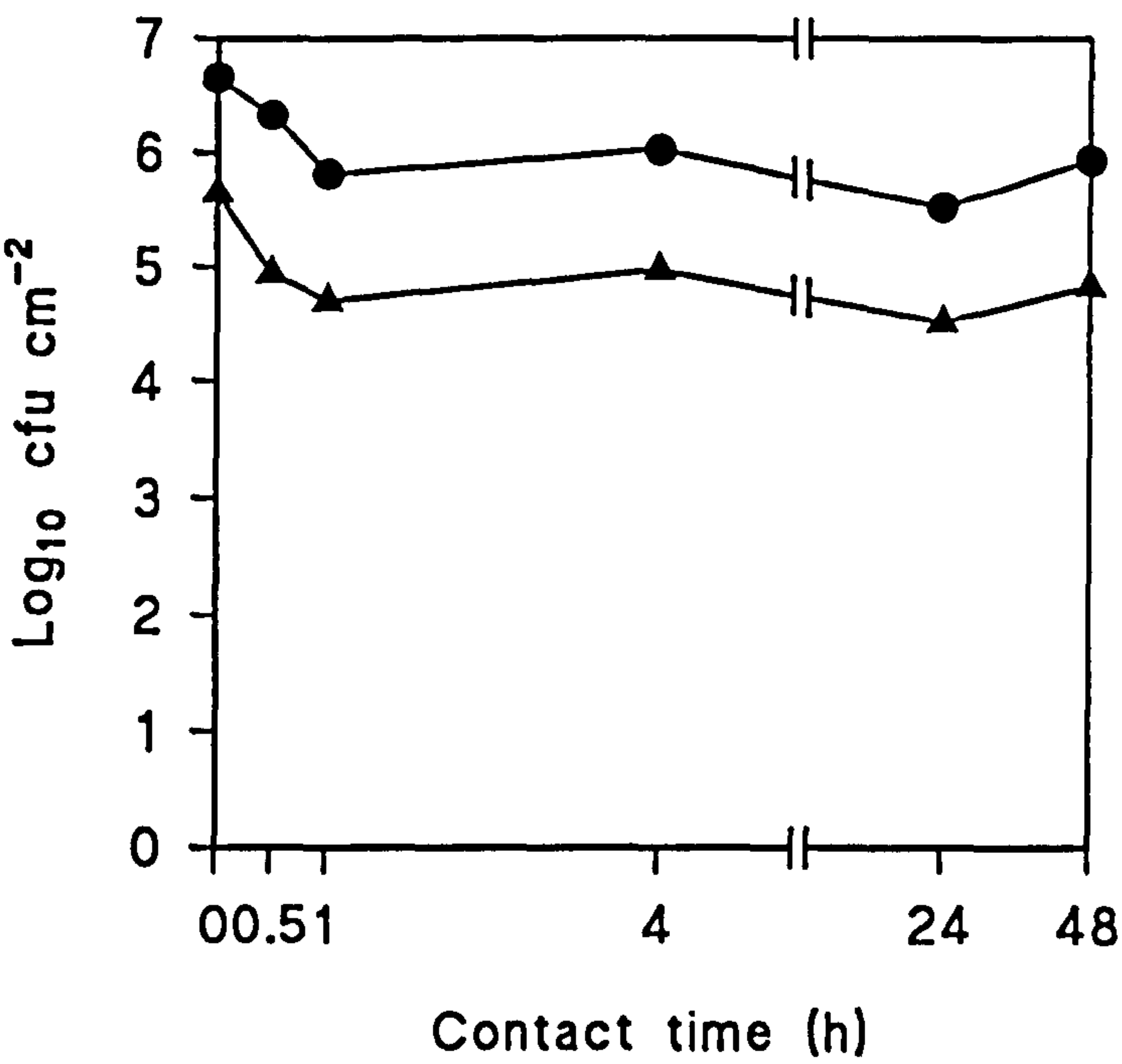
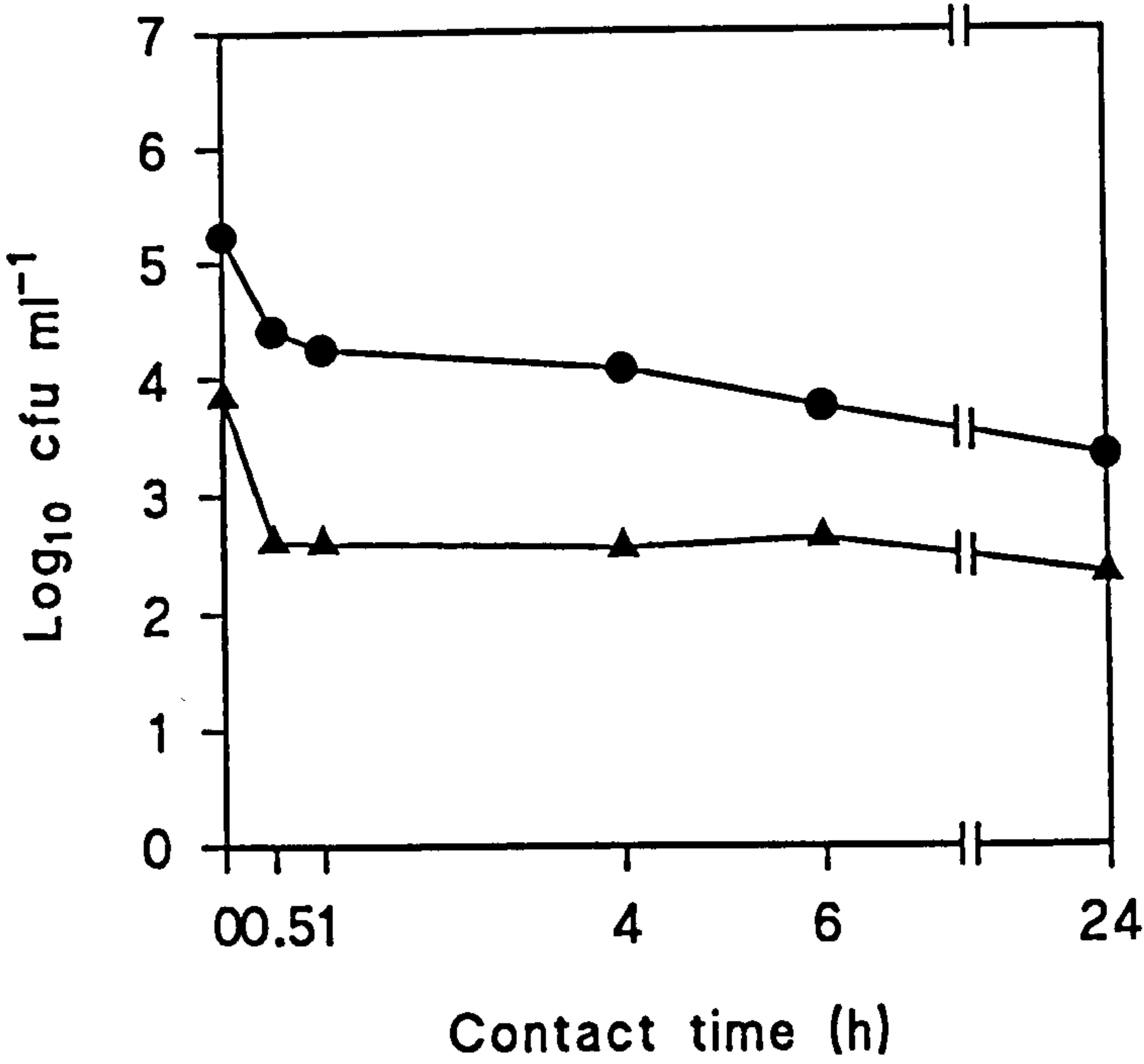


Figure 6.7 Effect of 400 mg/l GCO-30 (with Visigard) on total bacterial flora (●) and *L. pneumophila* (▲) in the planktonic phase.



WCO-130-30E (with Penetrex^R-type biodispersant)

At both concentrations of this biocide that were tested no bacteria could be recovered from the planktonic phase after 0.5 hour contact time (Figure 6.8 and 6.9). The bacteria did not recover from the inhibitory effects of the biocide and none were recoverable from the sample. The data suggested that in the aqueous phase the recommended concentrations of 300 and 600 mg/l were sufficient to control bacterial populations.

The biofilm populations were more resistant to biocide treatment than the planktonic phase. At a biocide concentration of 300 mg/l the numbers of viable bacteria recovered from the biofilm decreased to less than 10% within 3 hours contact time (Figure 6.10) but then stabilised at this value. However, there was some indication that the biocide might be selectively inhibitory to legionella since these numbers decreased by over 99% of their original value by 48 hours contact time.

The total flora was more susceptible to a concentration of 600 mg/l of WCO-130-30E (with biodispersant) and their numbers decreased by over 50-fold by 24 hours (Figure 6.11). Significantly, the legionellae were completely eradicated by 48 hours, showing that the biocide is selectively inhibiting legionellae or at least members of the biofilm microbial consortium which provide nutrients for the growth of legionellae in aquatic environments.

Figure 6.8 Effect of 300 mg/l WCO-130-30E (with biodispersant) on total bacterial flora (●) and *L. pneumophila* (▲) in the planktonic phase.

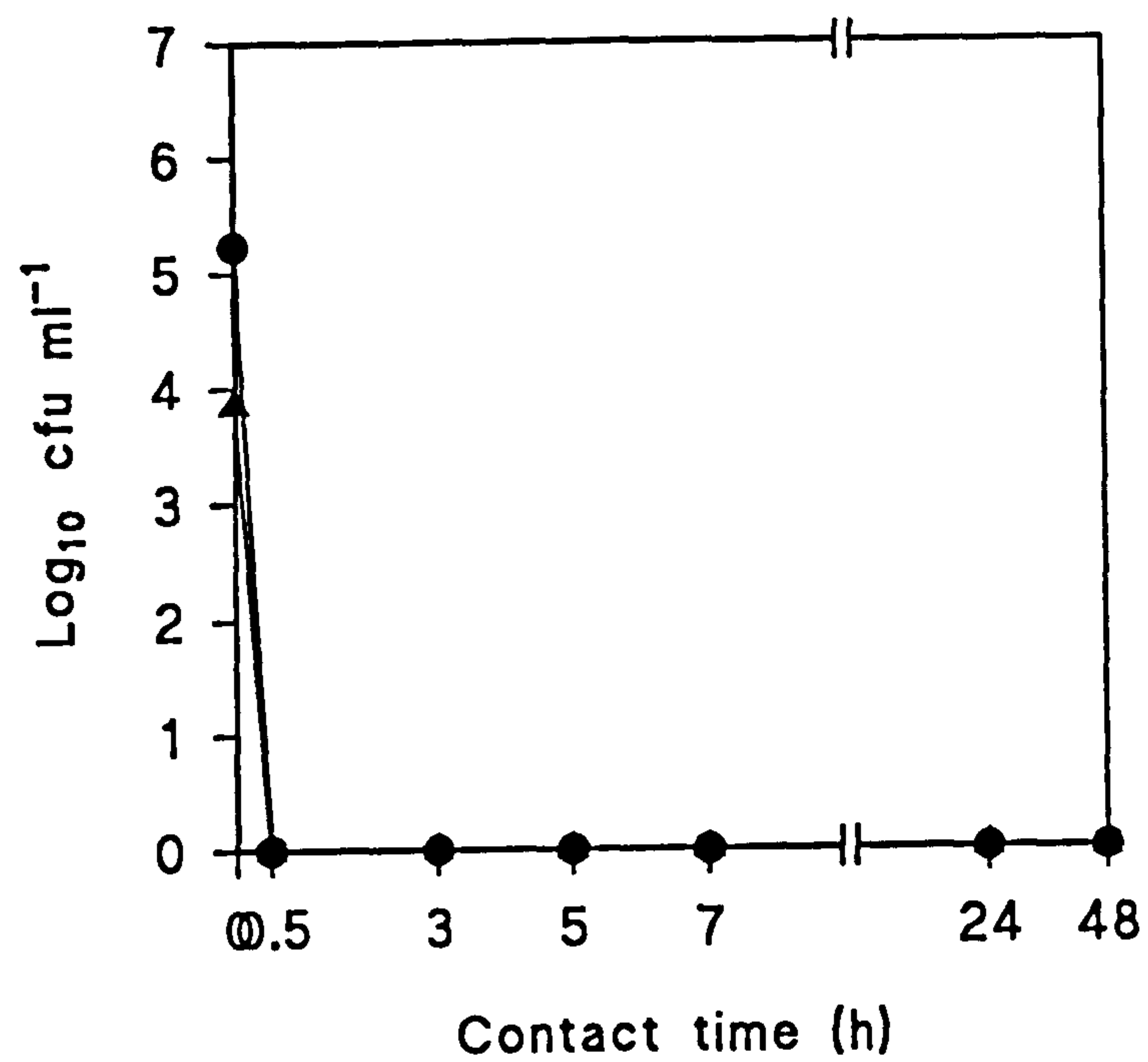


Figure 6.9. Effect of 600 mg/l WCO-130-30E (with Biodispersant) on total bacterial flora (●) and *L. pneumophila* (▲) in the planktonic phase.

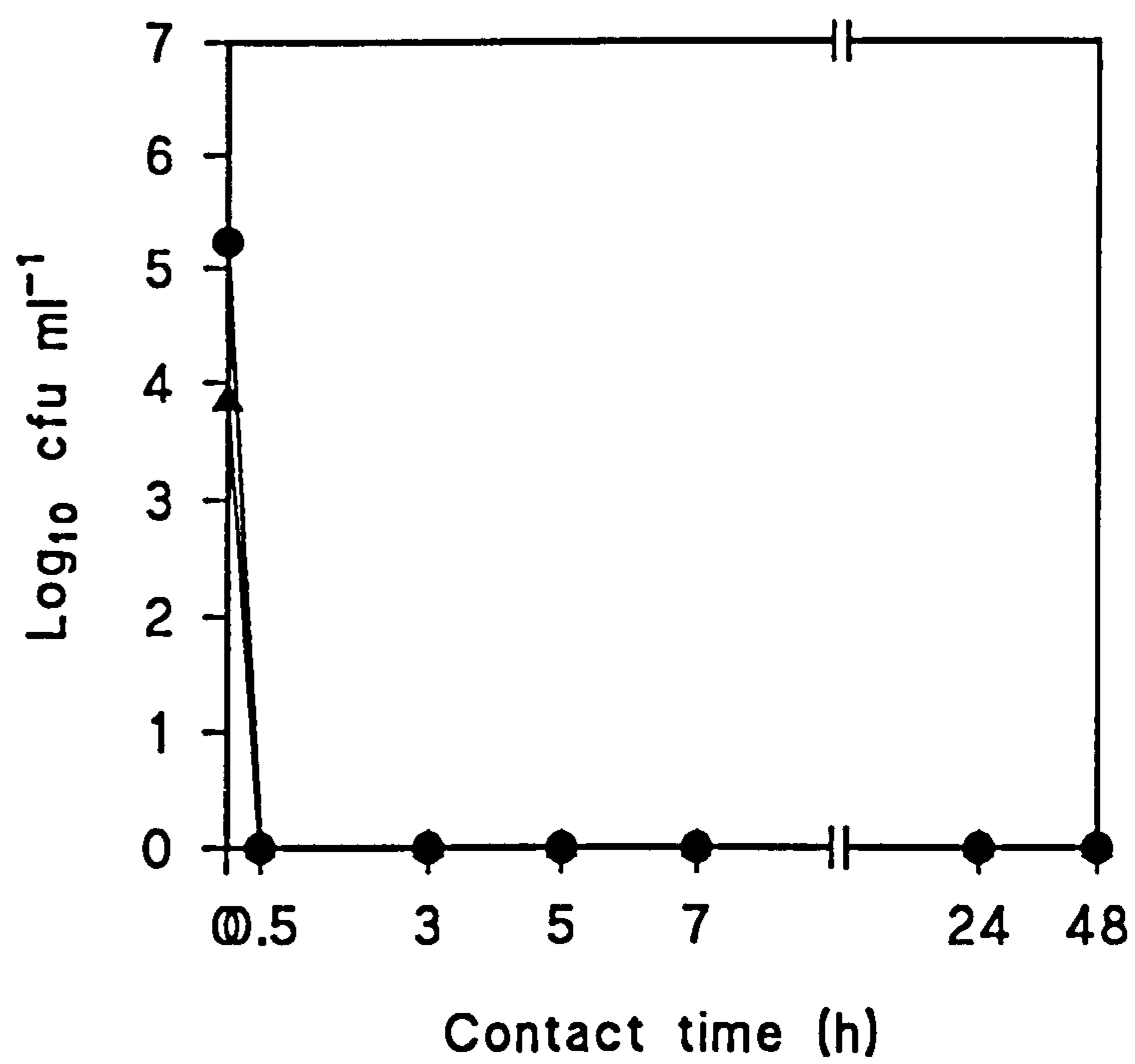


Figure 6.10 Effect of 300 mg/l WCO-130-30E (with biodispersant) on total bacterial flora (●) and *L. pneumophila* (▲) on biofilm developing on mild steel.

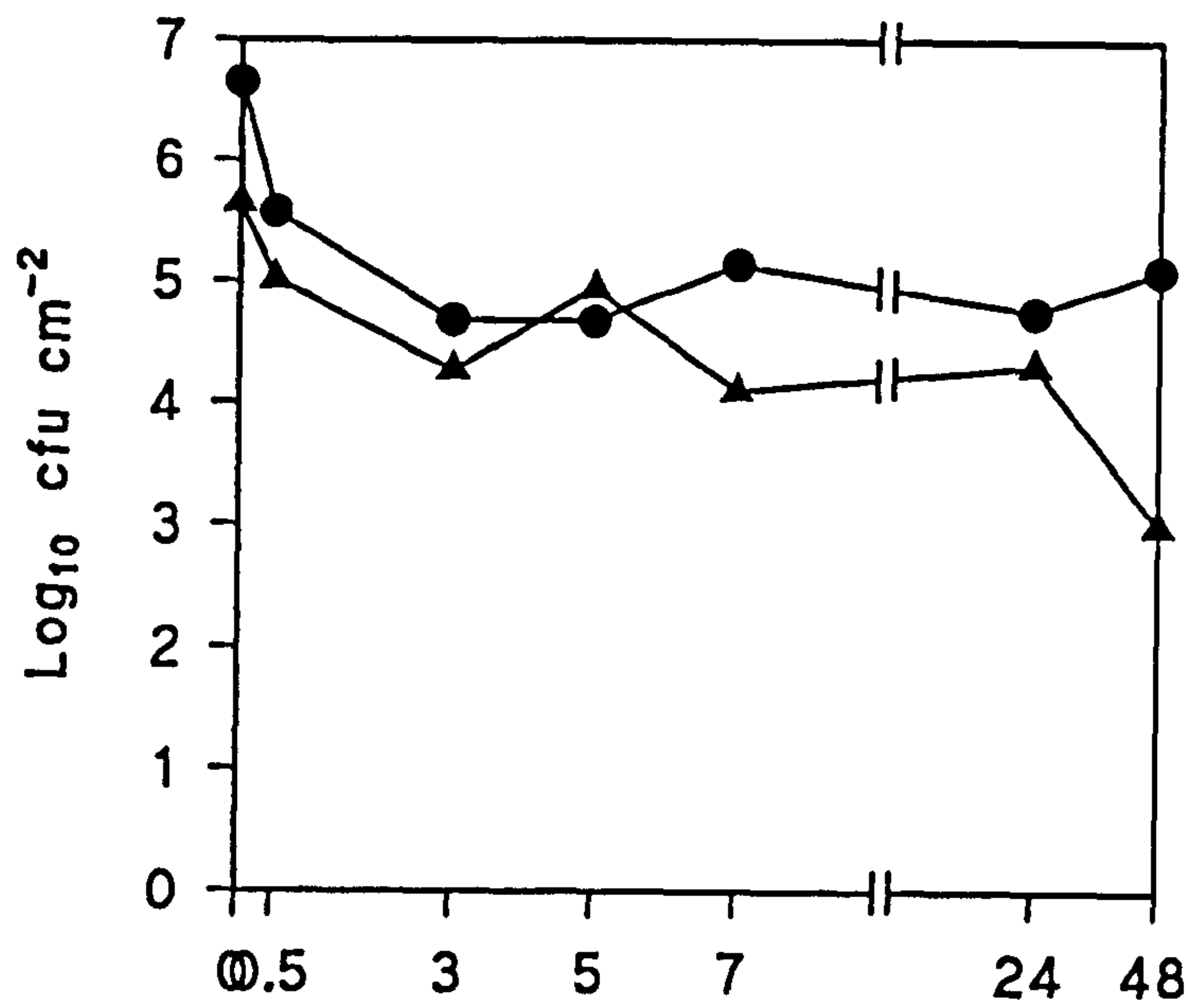
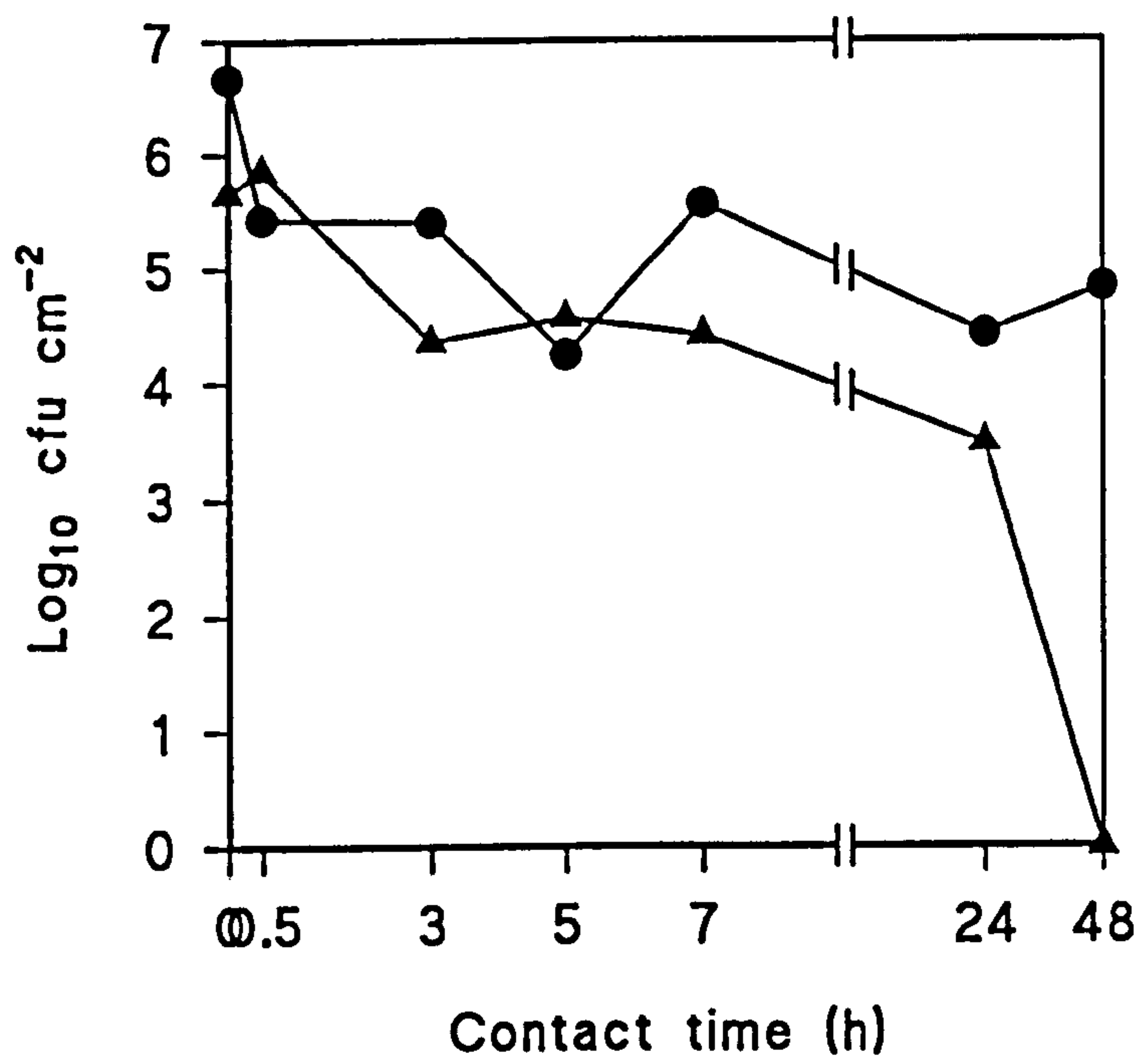


Figure 6.11 Effect of 600 mg/l WCO-130-30E (with biodispersant) on total bacterial flora (●) and *L. pneumophila* (▲) in biofilm developed on mild steel.



Dubact B^R with 10ppm Penetrex^R

Bromine and chlorine containing biocides are strong oxidising agents and have been shown to be beneficial at controlling aquatic bacteria at low concentrations. Little data is available on the efficacy against biofilm bacteria in controlled environments, and fears of biofilm presenting a permeability barrier have necessitated the inclusion of penetrating agents such as Penetrex^R to disrupt the film and aid biocide penetration. The ability of Dubact B^R with Penetrex^R to inhibit planktonic and sessile biofilm bacteria, including legionella, was therefore investigated using the chemostat model. At the recommended dose of 10 ppm there were no survivors in the planktonic phase within 1 hour contact time, in agreement with the commercial recommendation (Figure 6.12).

The total flora and legionella in the biofilm were also markedly inhibited at this concentration. The numbers of total flora decreased to 1.75×10^4 cfu cm⁻² a reduction of 99% in the first hour of contact (Figure 6.13). The number of *L. pneumophila* decreased by at least 99.9% of the original values after 7 hours with 2.35×10^2 cfu cm⁻². However, both legionella and other organisms remained within the biofilm following biocide treatment.

Figure 6.12 Effect of 10 mg/l Dubact B with 10 mg/l Penetrex on total bacterial flora (●) and *L. pneumophila* (▲) in the planktonic phase.

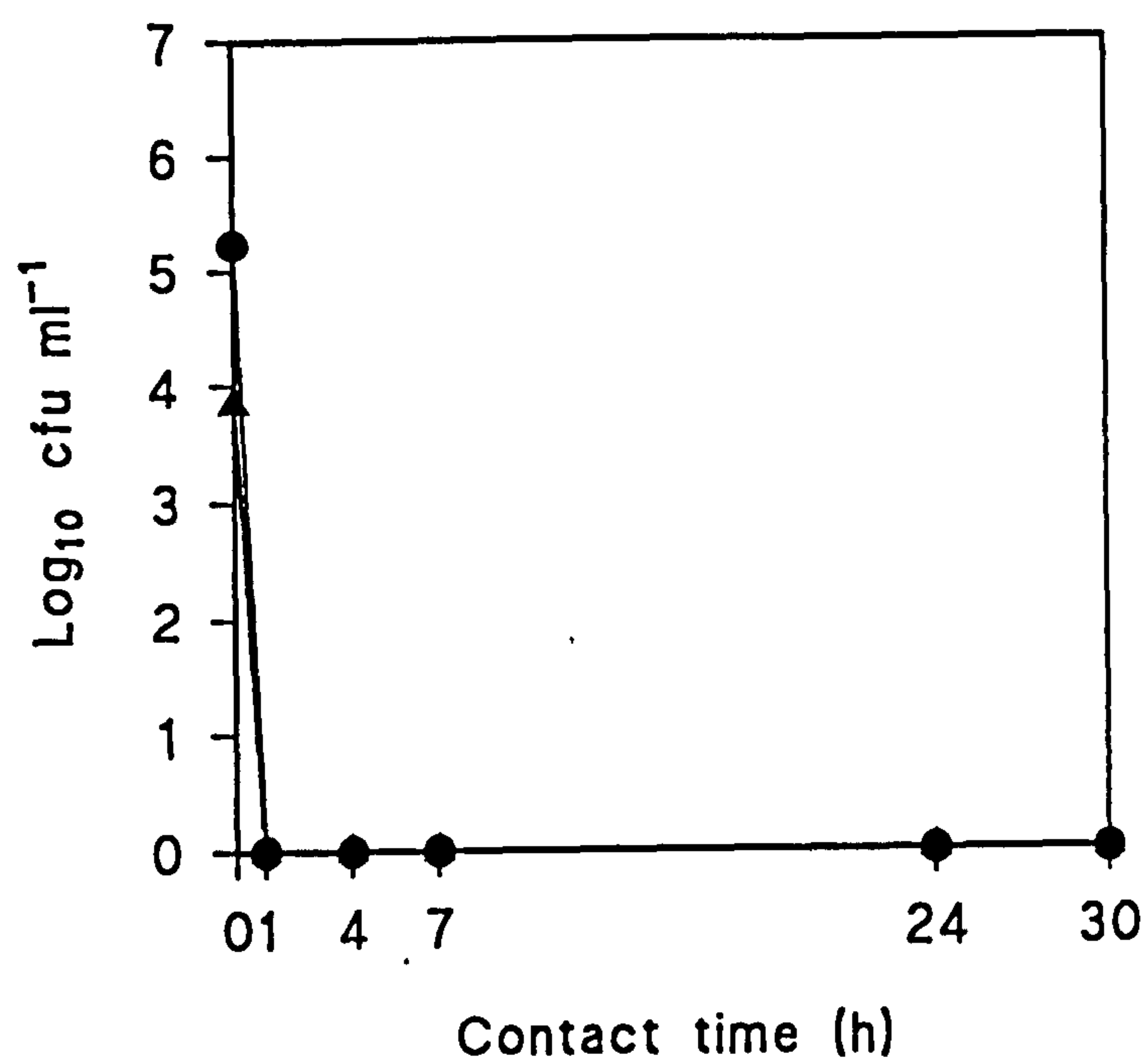
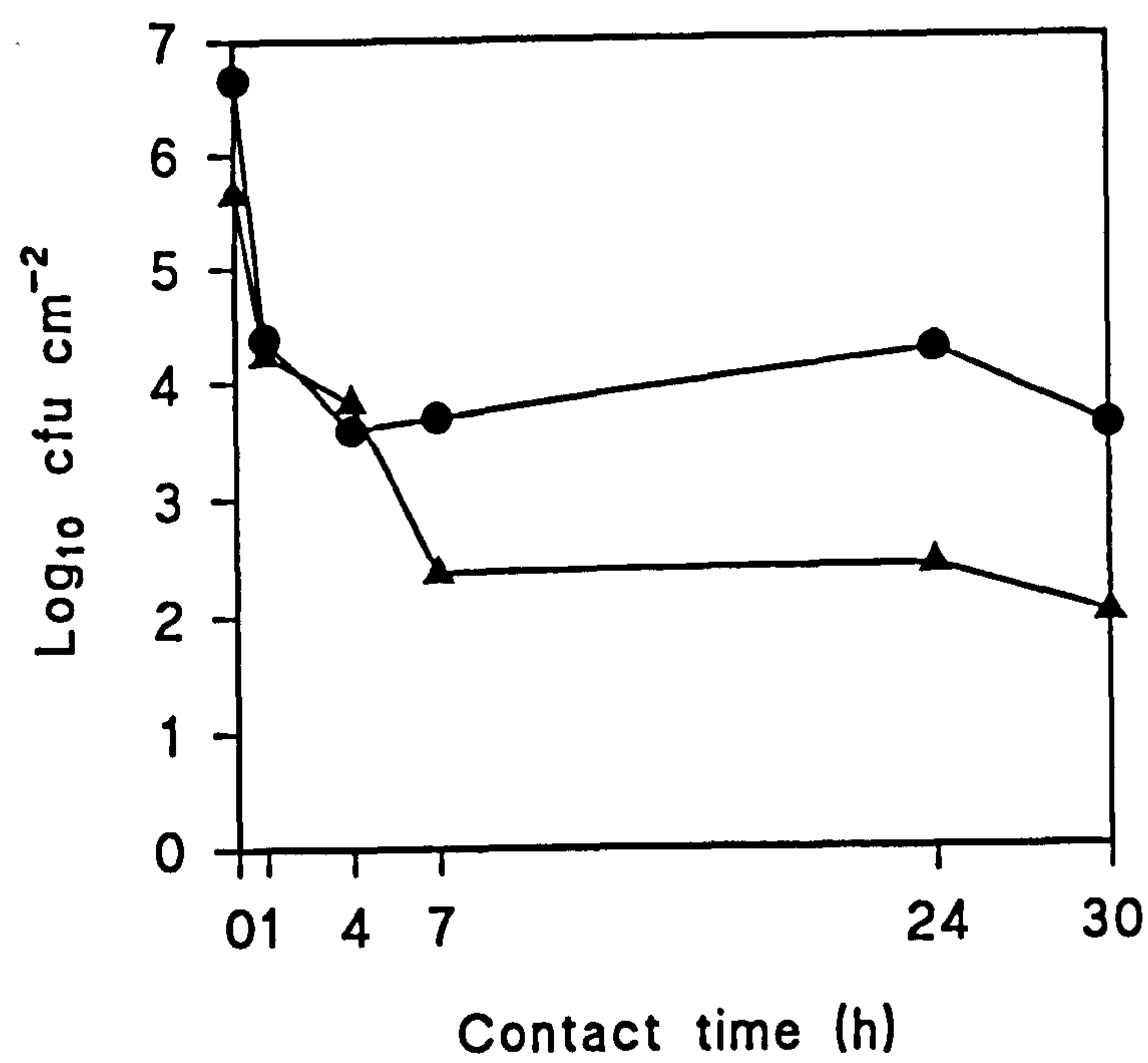


Figure 6.13 Effect of 10 mg/l Dubact B with 10 mg/l Penetrex on total bacterial flora (●) and *L. pneumophila* (▲) in biofilms developed on mild steel.



6.3.2 THE ANTIFOULING PROPERTIES OF A SILVER CONTAINING PAINT AND ITS POTENTIAL TO INHIBIT COLONISATION OF *L. PNEUMOPHILA*.

6.3.2.1 The composition of the planktonic phase

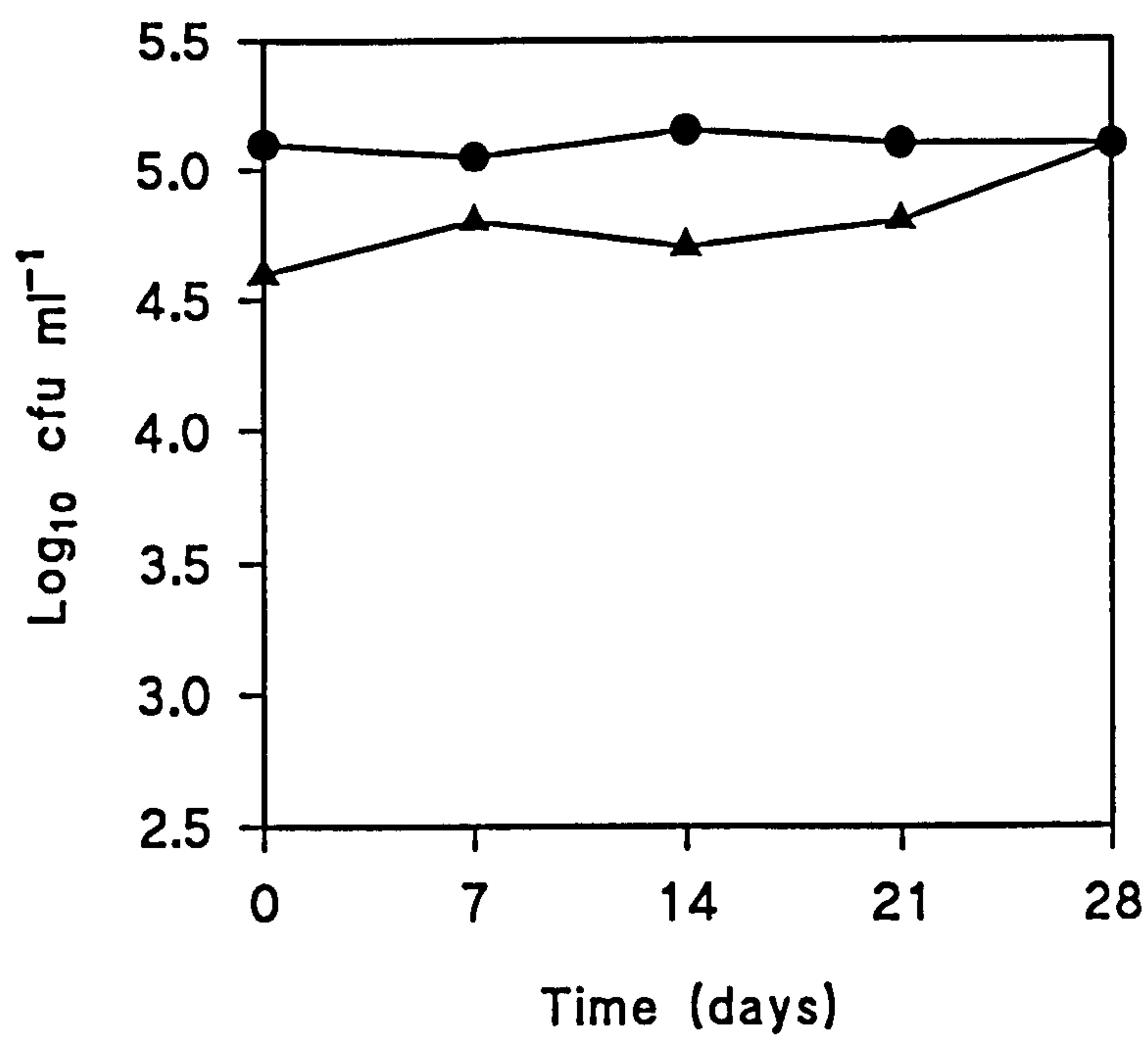
Prior to the inclusion of silver material into the third vessel the planktonic phase was similar, both in numbers and in composition, to that found in the first and second vessels. The total number of micro-organisms was constant at approximately 1×10^4 cfu ml⁻¹ and the legionella represented approximately 1% of this total (Figure 6.14).

Within the planktonic phase *Pseudomonas spp.* were the most abundant organisms.

When silver was added to the third vessel, the numbers of micro-organisms in the culture in the second vessel, which contained no silver surfaces, remained at a constant level of approximately 1×10^4 cfu ml⁻¹. The number of *L. pneumophila* also remained stable at about 1% of the total number of micro-organisms.

When silver painted tiles were inserted into the third chemostat vessel, the numbers of planktonic bacteria initially declined to 3.6×10^3 cfu ml⁻¹. Representing a reduction of 70% of the original planktonic micro-organisms and this suppression of growth was maintained up until 21 days. After this time, the number of micro-organisms gradually increased to a level similar to that found in the vessels containing no silver painted surfaces. The initial decrease in the total bacterial numbers was also accompanied by a similar loss in the numbers of *L. pneumophila*. Initially the numbers of legionella were reduced to only 6% of that occurring in the vessels containing no inhibitory surfaces.

Figure 6.14 Total numbers of planktonic bacteria in the presence (▲) and absence (●) of silver painted tiles.



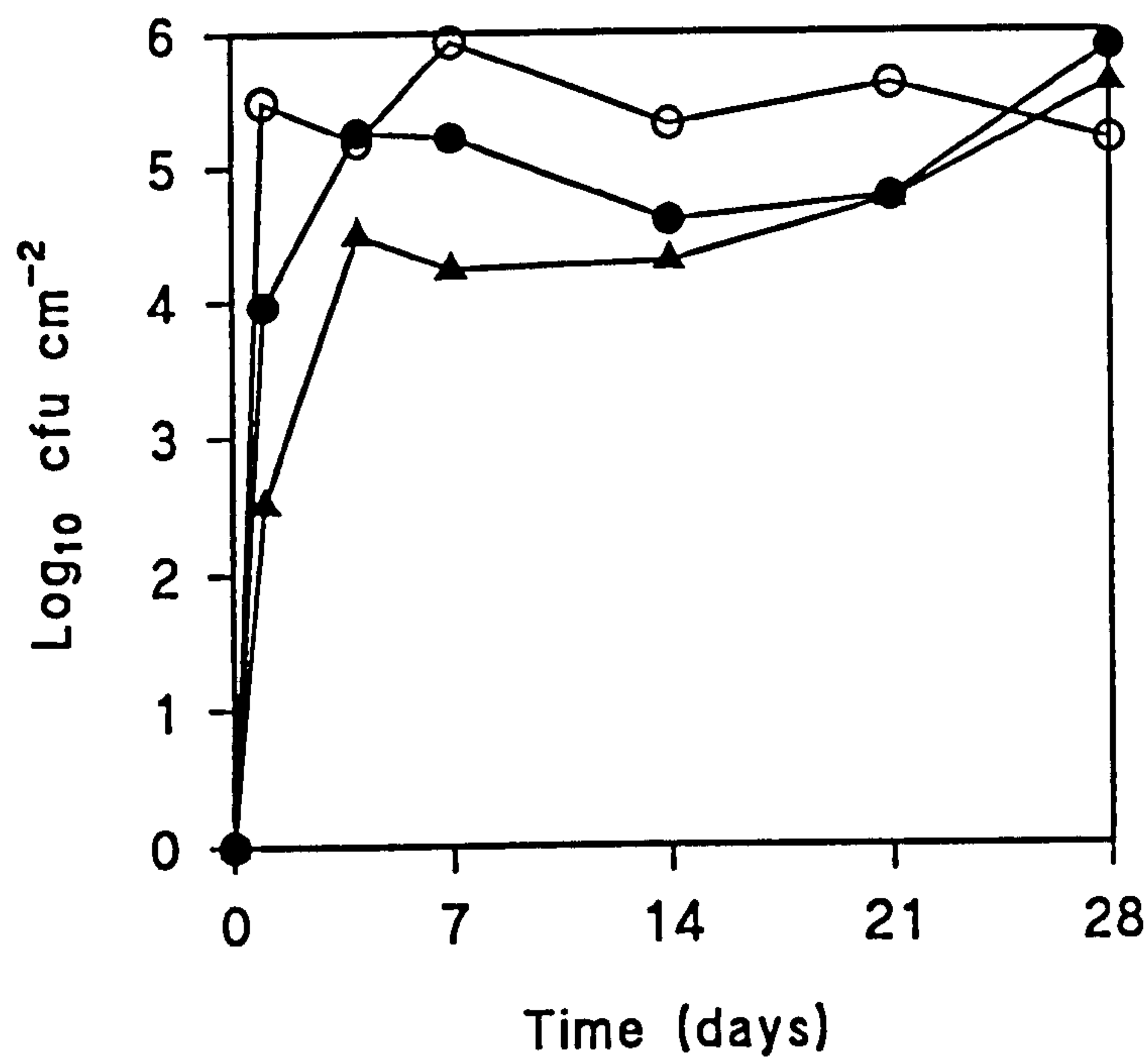
6.3.2.2 Colonisation of the surface

Colonisation of the glass surfaces in the absence of the silver paint was very rapid, reaching 3.05×10^5 cfu cm⁻² after only 24 hours (Figure 6.15). This high level of colonisation remained stable through out the experiment with only a small reduction on the 28 day biofilm.

Colonisation of the silver painted surfaces proceeded at a greatly reduce rate compared to that of the glass control. After 24 hours the total number of micro-organisms had reached 3.25×10^2 cfu cm⁻²; only 0.1% of the colonisation that occurred on the control glass surfaces over the same time period. The numbers of bacteria in the biofilm remained at approximately 3×10^4 cfu cm⁻² after 4 days and then maintained this level up until 21 days. The extent of biofouling was reduced by 90% of that occurring on the control glass surface for the first 21 days. At 28 days the advantage of the silver paint in preventing colonisation was negated since the total number of bacteria incorporated into the biofilm on silver increased to 4×10^5 cfu cm⁻², 2.5 times more than that which occurred on the control glass.

The initial colonisation of glass tiles suspended in the chemostat simultaneously with the silver painted tiles was slower than that when silver was absent. Biofouling was greater than on the silver paint itself, and biofilm formation was intermediate between that on the glass control and that on the paint. After 24 hours, the number of micro-organisms had reached 9.1×10^3 cfu cm⁻², 34 times less than the biofilm on the control glass surface and 30 times more than that on the paint. The numbers of micro-organisms stabilised after 4 days at approximately 6×10^4 cfu cm⁻². At 28 days the numbers increased to 7.65×10^5 , which was higher than that on the control glass surfaces.

6.15 Colonisation by total flora of untreated glass in the absence of silver paint (O), glass with silver paint (●) and silver painted glass (▲).



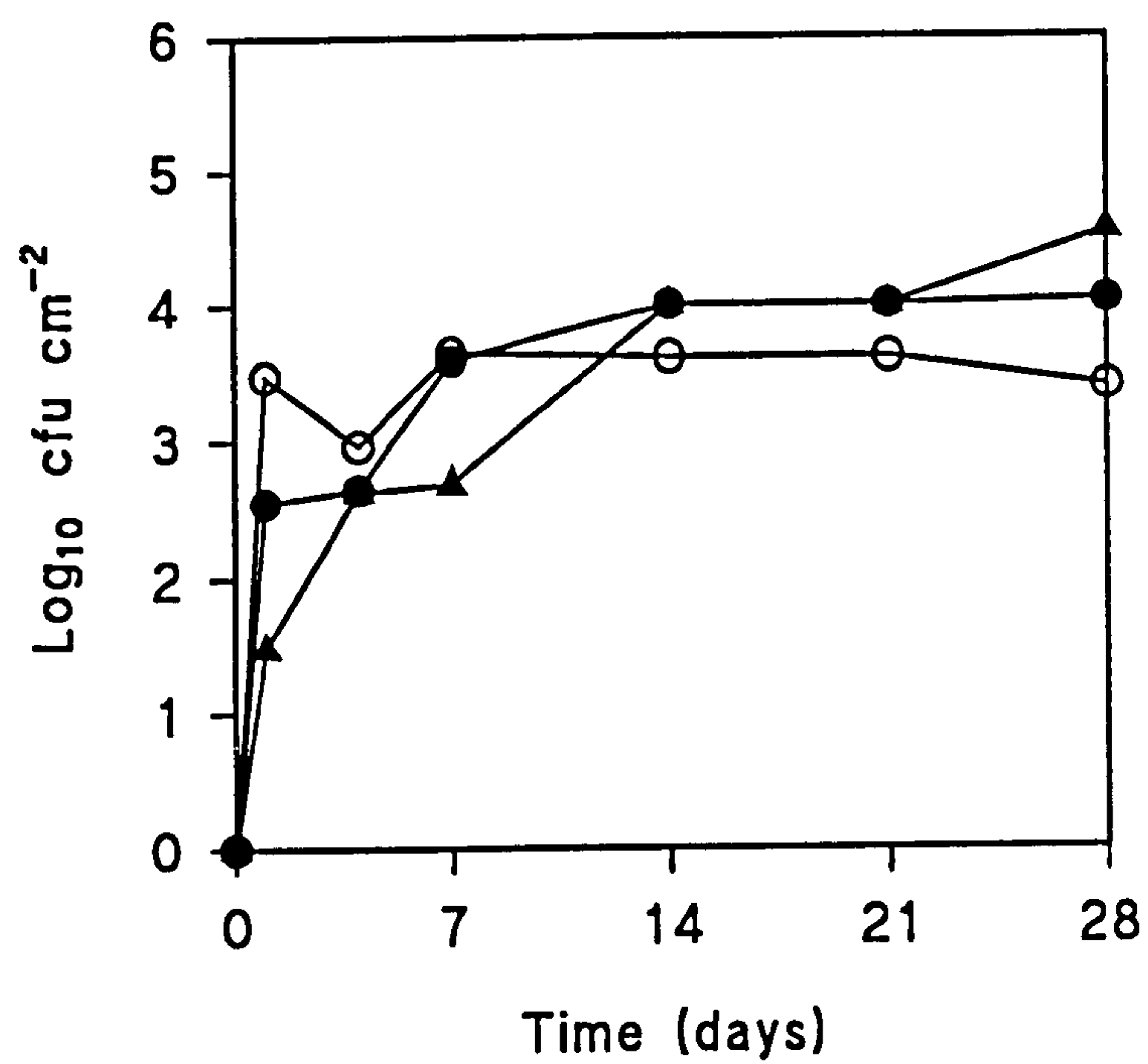
6.3.2.3 The incorporation of *L. pneumophila* into the biofilms

In the absence of silver paint the incorporation of legionella into biofilms on glass surfaces was initially very rapid (Figure 6.16). After 24 hours the number of legionella was 3×10^3 cfu cm⁻². The level dropped slightly at 4 days but otherwise remained at approximately 4×10^3 cfu cm⁻² for the 28 day trial.

The inclusion of legionella into the biofilm on the silver paint proceeded at a slower rate than on the glass control. After 24 hours the numbers of legionella had only reached 30 cfu cm⁻², 1% of the numbers occurring on the control glass tiles. After 4 days the numbers stabilised at approximately 3×10^3 cm⁻² and maintained a lower number than on the glass controls. The silver paint reduced the numbers of legionella by 25% over a 21 day period. However, the number of *L. pneumophila* began to increase after 14 days and by 28 days they had reached 3.5×10^4 cfu cm⁻², an increase of 70% of the numbers that occurred on the glass surfaces in the absence of silver.

The glass surfaces that were alongside the silver painted tiles in the third vessel also showed an initial reduction in the colonisation rate by the legionella. After 4 days the number of legionella on the glass surfaces were similar to the number on the control surface until 21 days. After 21 days the numbers of legionella on the glass increased to 5 times the control number.

Fig 6.16 Colonisation by *Legionellae* of untreated glass in the absence of silver paint (O), glass in the presence of silver paint (●) and silver painted glass (▲).



6.3.2.4 The population profiles of biofilm development in the presence of silver paint.

The pioneer species on the silver paint were *Methylobacterium* spp. these organisms comprised 50 % of the total population on the silver surface (Table 6.2). The other species present included *P. maltophilia*, *P. mendocina*, *P. stutzeri*, Actinomycetes, *Flavobacterium* spp. and *Acinetobacter* spp. The surface was then colonised by *P. paucimobilis*, *P. vesicularis* and *Alcaligenes* spp. after 4 days, possibly after the more tolerant species had formed a protective layer of cells on which these species could grow. After 28 days the biofilm contained a diverse range of microorganisms in which the pseudomonads were most abundant.

The 1 day glass biofilm inserted with the silver painted surface contained a greater diversity of microorganisms than the silver painted tile (Table 6.3). The flora was dominated by *Methylobacterium* spp. as was that on the silver surface, with 5.7×10^3 cfu cm⁻². The pseudomonads remained predominant over the whole of the colonisation period, the most commonly occurring species being *P. maltophilia*, *P. mendocina*, *P. paucimobilis* and *P. vesicularis*. The other species that remained within the biofilm for the majority of the test included, *Alcaligenes* spp., *Flavobacterium* spp. and *Acinetobacter* spp. One organism was cultured from biofilms samples on the initial GVPC/BCYE isolation plate but could not be subcultured. The organism is listed in the population profiles as OGN (other gram negative organisms).

Table 6.2 Population profiles on silver painted glass surfaces.

	Age of biofilm					
	day 1	day 4	day 7	day 14	day 21	day28
P.fluorescence						60
P.maltophilia	0.2	4.1	1	3		100
P.mendocina	0.2		17		12	
P.paucimobilis		21	30	80	220	150
P.stutzeri/cdc .vb3	0.2	40	60	20	20	
P.testosteroni						500
P.vesicularis		30	69	30	180	700
P.xylesoxidans						850
Actinomyces	0.5	45	3	10	9.6	20
Alcaligenes		2	60	20		20
Flavobacterium	0.6	1	1	20	0.8	50
Methylobacterium	3	1	10	110	40	620
Klebsiella				0.3		
Acinetobacter	0.6	190	70	2.6	13	440
Unidentified Gram negatives	0.4	0.2	2		40	
L. pneumophila	0.3	4.3	4.9	7.6	99	350

Numbers are expressed as cfu cm⁻² x10³. Numbers of non-legionella populations are represented as a sum of those cfu cm⁻² occurring on R2A, BCYE and GVPC media.

Table 6.3 Population profiles on glass in the presence of silver painted glass.

	Age of biofilm					
	day 1	day 4	day 7	day 14	day 21	day28
<i>P.acidovorans</i>		800		50	40	120
<i>P.maltophilia</i>	9	100	400	60	30	1700
<i>P.mendocina</i>	4		100	50	400	
<i>P.paucimobilis</i>	1	760	300	130	200	820
<i>P.stutzeri/cdc .vb3</i>	21		400	20		
<i>P.testosteroni</i>						400
<i>P.vesicularis</i>	9	2100	1300		110	900
<i>P.xylesoxidans</i>	4					2500
<i>Actinomyces</i>	0.1			110	20	
<i>Alcaligenes</i>		700	200	80	20	10
<i>Flavobacterium</i>	1.8	20	20	10	3	130
<i>Methylobacterium</i>	57	300	100	10		600
<i>Acinetobacter</i>			500	70	510	540
Unidentified Gram negatives	18		90	20	60	100
<i>L. pneumophila</i>	1.8	4.5	3.85	28	159	108

Numbers are expressed as cfu cm⁻² x10³. Numbers of non-legionella populations are represented as a sum of those cfu cm⁻² occurring on R2A, BCYE and GVPC media.

6.3.2.5 Scanning electron microscopy

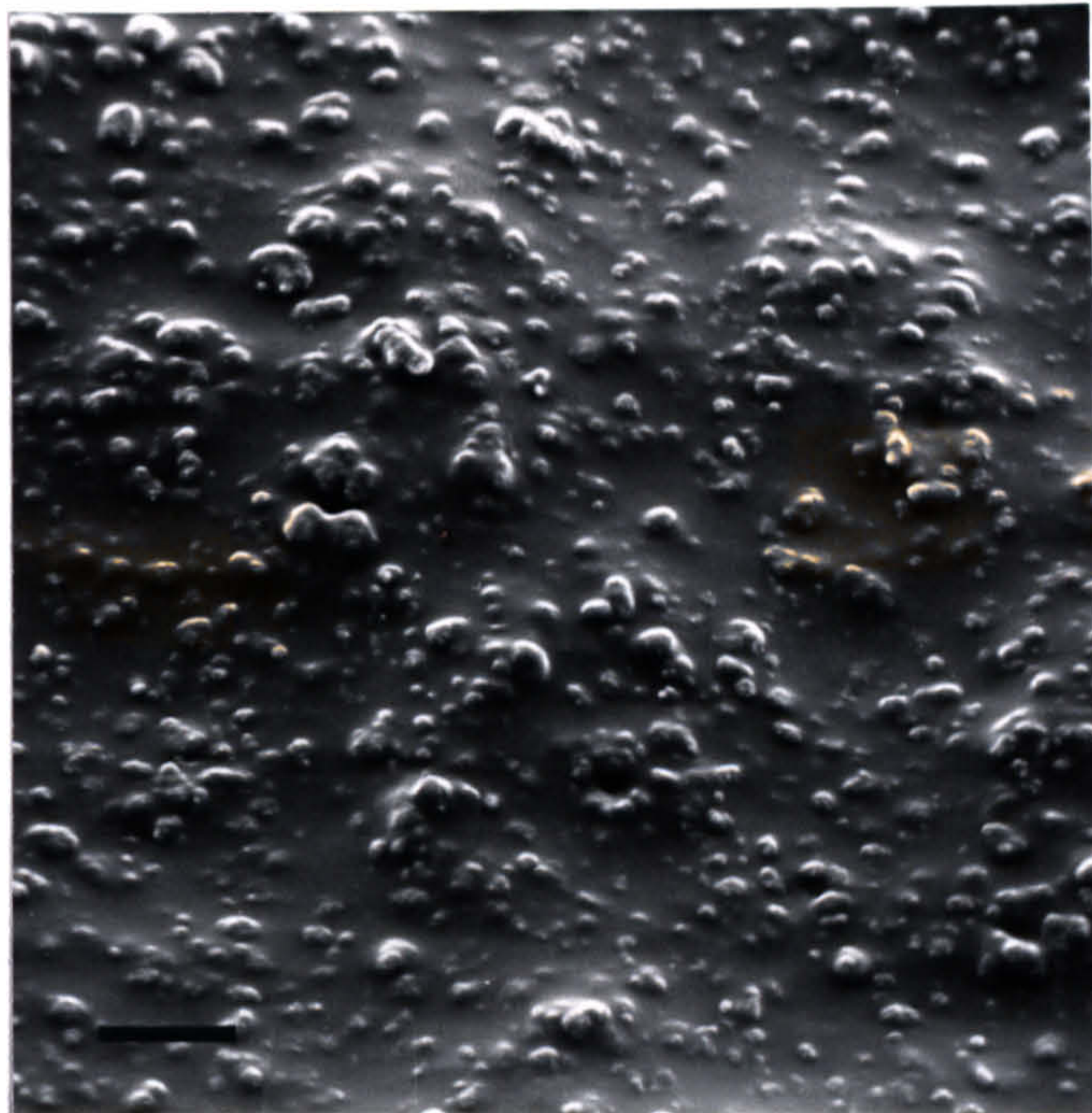
Scanning electron micrographs showed that the glass controls in the absence of silver paint were heavily colonised after 21 days (Figure 6.17). The morphology of the microorganisms attaching was varied with many types of short and long rods and cocci. Many microcolonies with extracellular material reminiscent of polysaccharide were evident.

Silver painted surfaces appeared very granular, presumably due to particles of silver embedded in the paint. Occasionally, there were a few long, thin rods detectable but there was little other evidence of pronounced colonisation (Figure 6.18).

In the presence of the painted tiles, glass surfaces had very localised colonisation with predominantly large, short rods. Large areas of the surfaces were not colonised.

Figure 6.17 (a) Scanning electron micrographs showing the silver painted surface prior to inclusion into the chemostat

(a)



(b & c) the glass control surface showed lower colonisation than expected in the presence of the silver paint 21 days old.



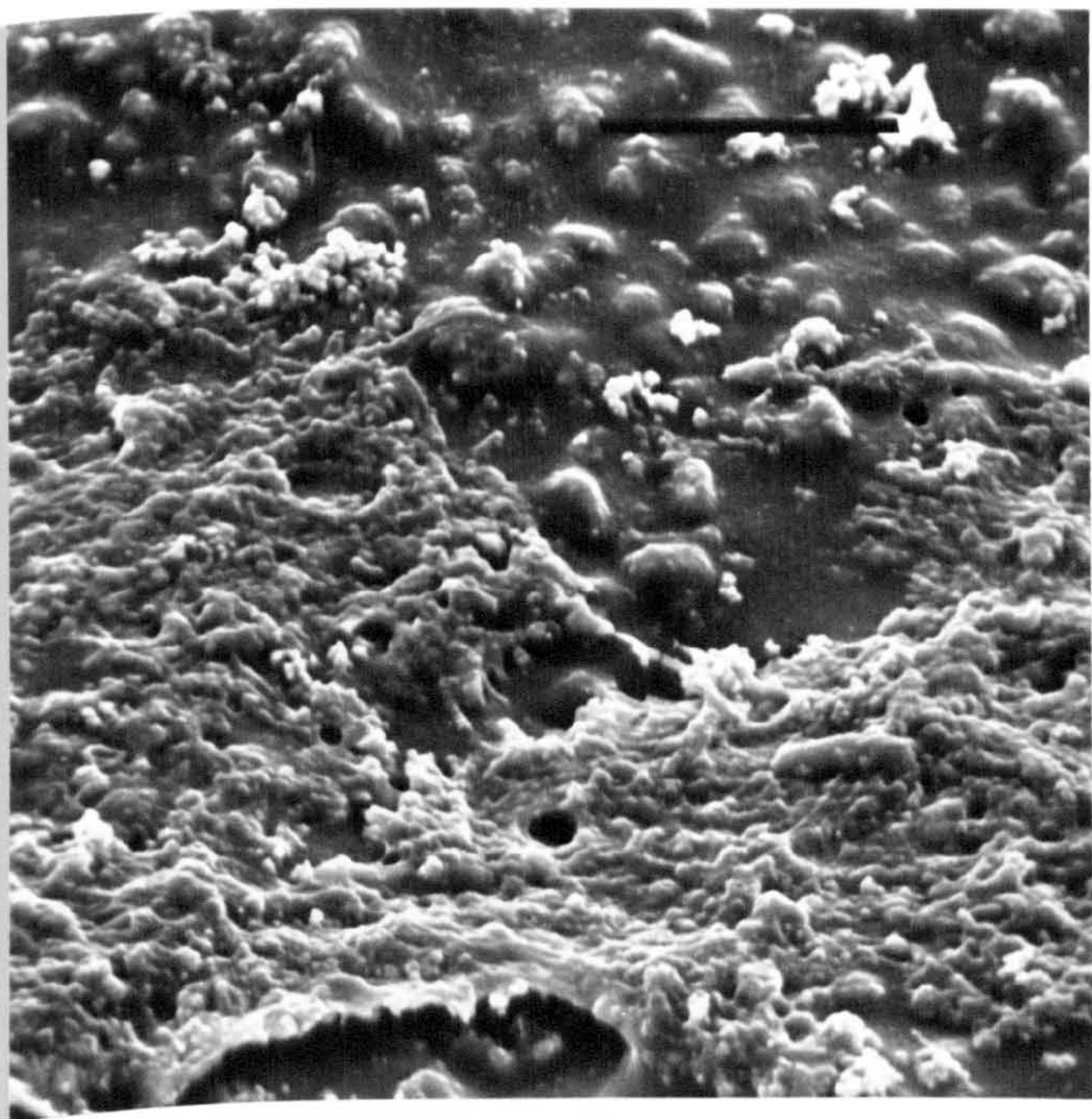
(b)



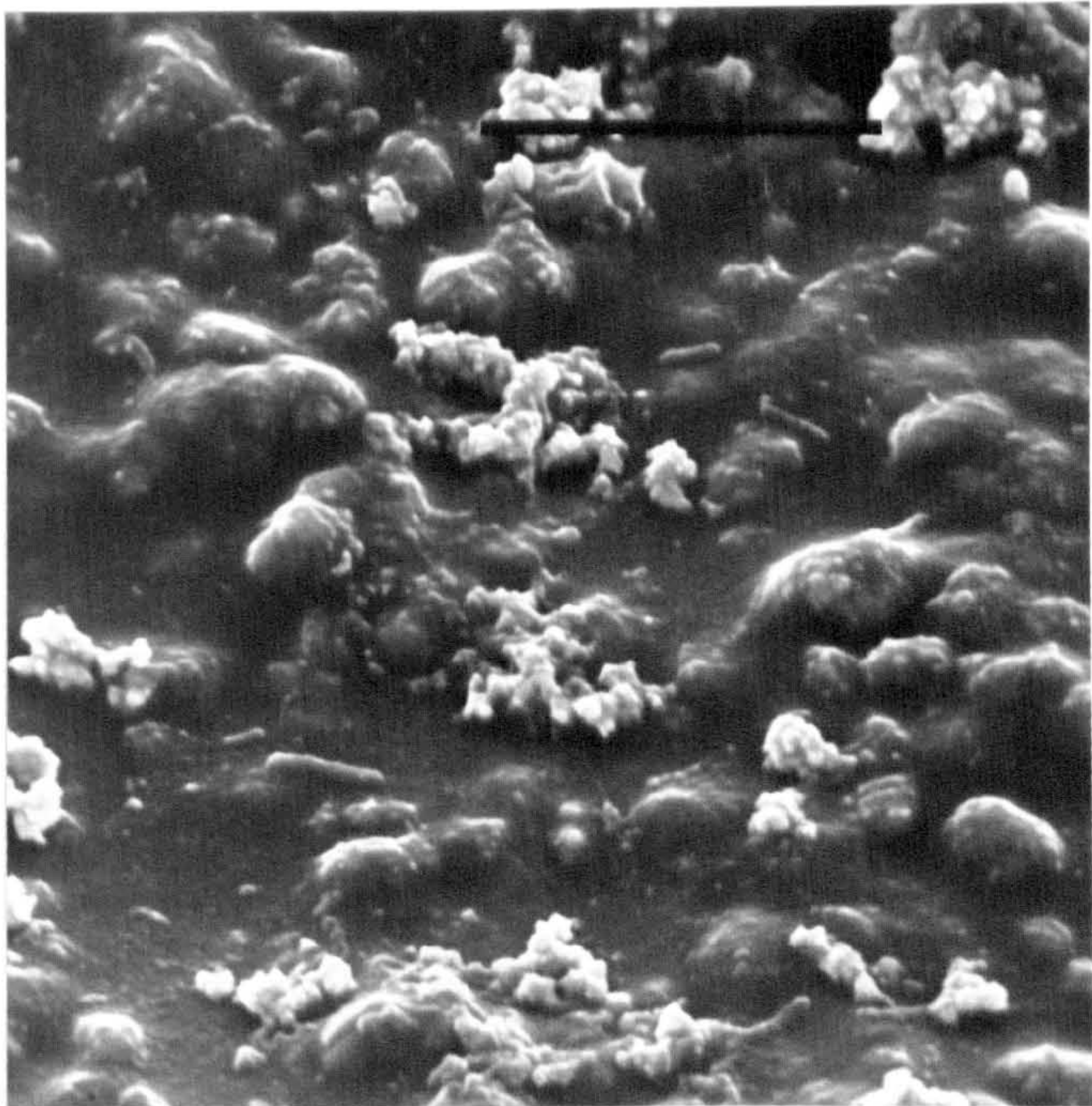
(c)

Figure 6.18 scanning electron micrographs of the silver paint after 21 days showing that biofilm had developed (a), where bacteria could be observed (b). Crystal structure could be seen to have developed on the paint surface (c) and these were colonised by bacteria (d).

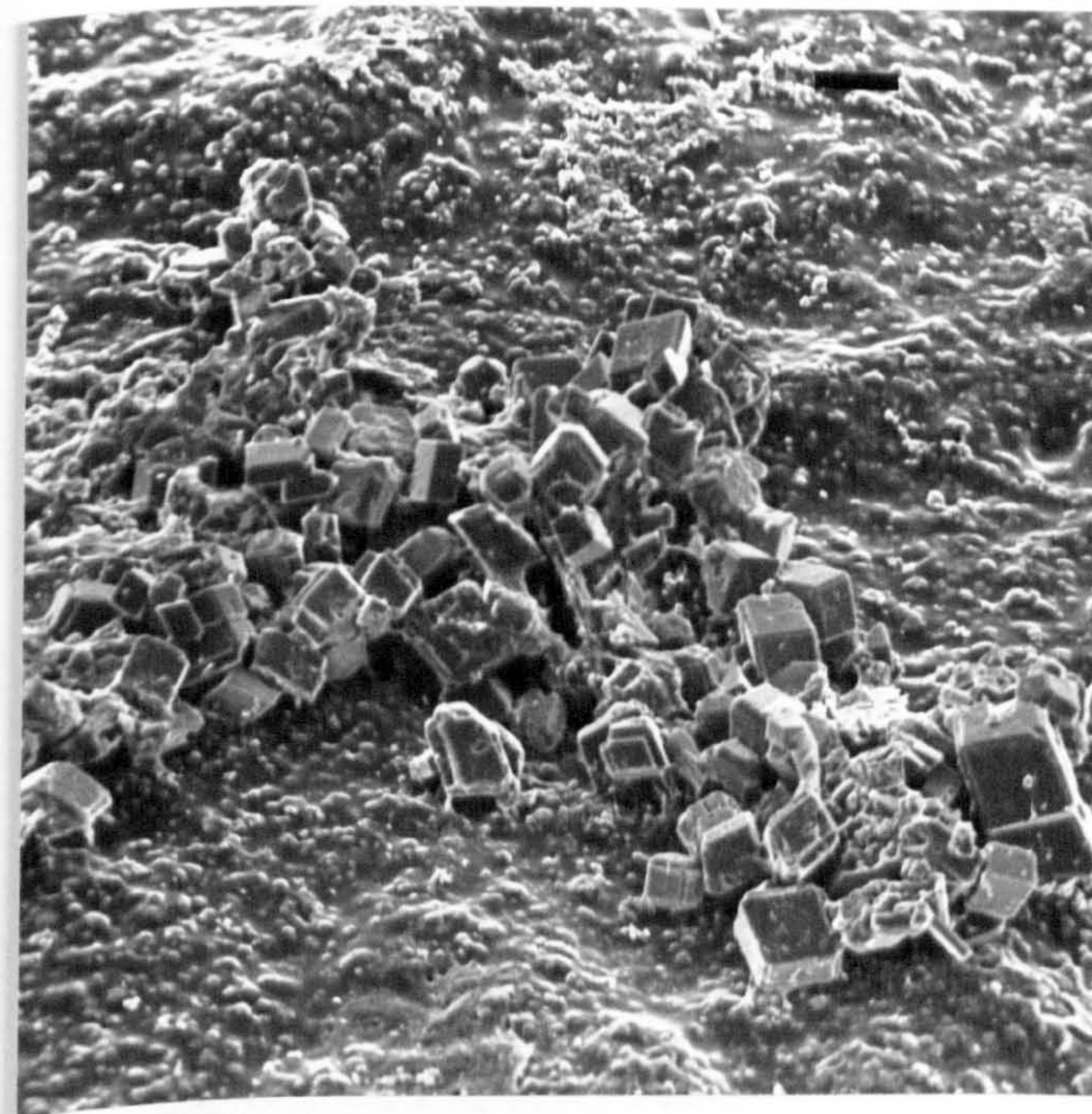
(a)



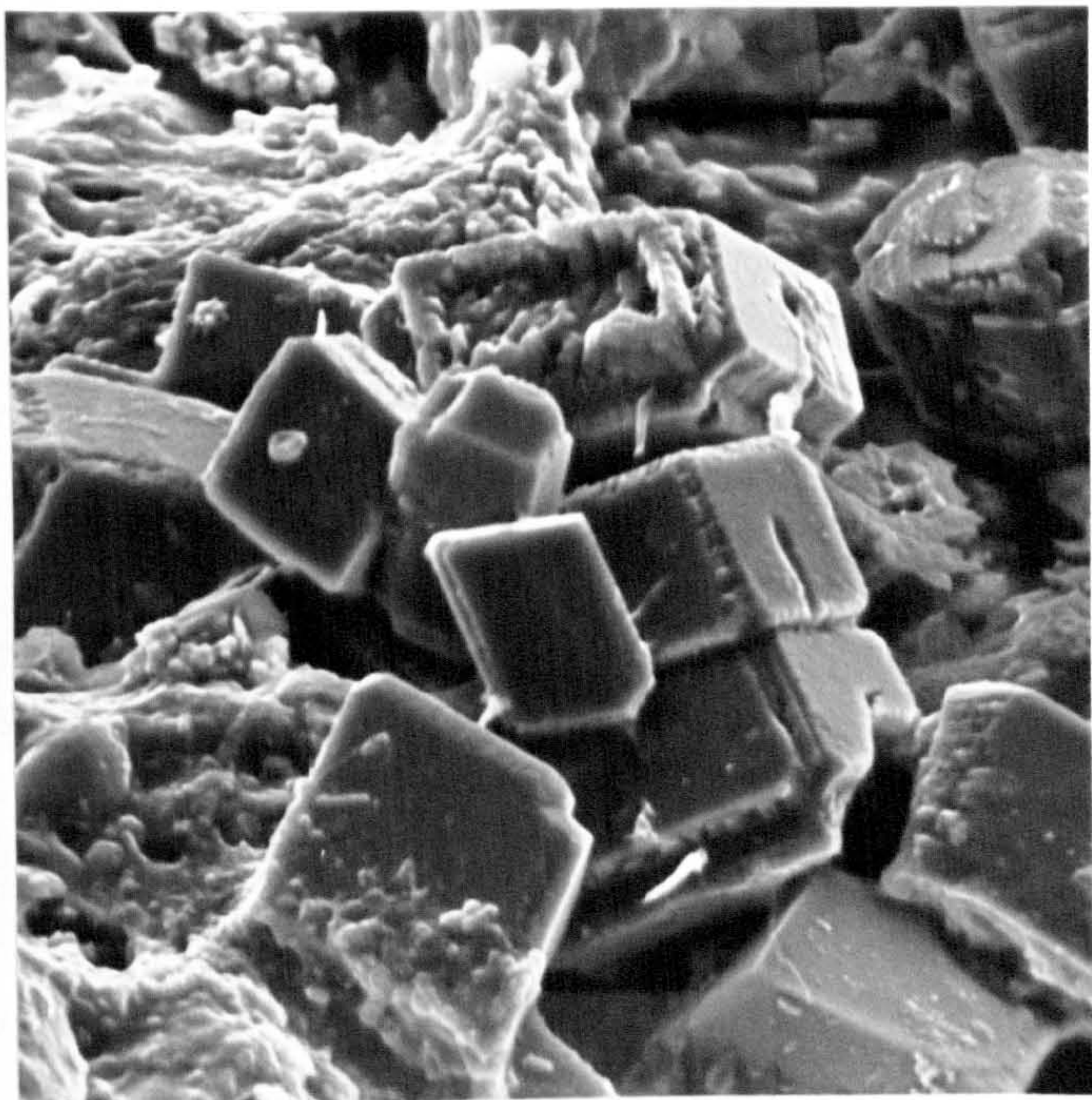
(b)



(c)



(d)



6.4 DISCUSSION

The biofilm was found to be more resistant to biocide than the planktonic phase and protected the *L. pneumophila* from biocide treatment. Viable legionella could be recovered from biofilm samples (and some planktonic samples) following biocide treatment using the manufacturers recommended dose and contact times. This implies that some treatments currently available are ineffective in controlling *L. pneumophila*.

In the planktonic phase, two of the biocides, WCO-130-30E & Dubact B^R were able to completely inhibit growth of flora including *L. pneumophila*. The third biocide GCO-30 had no inhibitory effect on the total viable count of bacterial flora and *L. pneumophila*.

The non-oxidising biocides were not effective against biofilm, although WCO-130-30E did remove the *L. pneumophila* but excessively high concentrations of biocide and long contact times were required. This biocide appeared to selectively reduce the population of *L. pneumophila*.

Biocides that effectively removed planktonic flora completely could be used to control *L. pneumophila* in water systems if biocide was added continually since colonisation would be prevented. However, If biocide treatment was not continuous, biofouling of the system would occur and removal would require alternative treatments.

Neutralisers were not added to the biofilm and planktonic samples prior to inoculation on to agar media since no one neutraliser would be available for all biocide treatments. However, the failure of the biocides to remove both legionellae and non-legionellae populations would indicate these would have been unnecessary.

The silver painted surface was able to retard initial colonisation and growth of the complex aquatic community for up to 21 days. Biofilm then began to develop, suggesting loss of inhibitory activity, possibly due to the selection of a silver tolerant population. More likely is the gradual leaching of silver in to the aqueous phase resulting in depletion from the surface. Bacteria were subsequently able to tolerate the reduced concentrations and form biofilm. The hypothesis that there is gradual loss of silver ions from the paint is supported by the reduction in the numbers of both the total planktonic micro-organisms and the sessile biofilm population on glass suspended alongside the painted surfaces. This reduced colonisation also implies that untreated surfaces within a water system could be afforded additional protection from colonisation by leached silver. The concentration of silver measured at this time in the aqueous phase was below the detection limit of 1 µg/l. After 21 days the numbers of microorganisms on the surface of the glass tile in the presence of the silver painted tile recovered to expected values, suggesting that silver was no longer leaching from the surface of the paint.

The colonisation of the painted surface by legionella was initially very low. However, after 14 days the legionella were no longer inhibited. Possibly due to sufficient leaching of the silver from the surface to allow the development of a sufficiently mature biofilm to sustain the complex nutritional requirements of the legionella.

After 21 days the colonisation on the silver paint is actually enhanced over that of the untreated control, suggesting some component of the paint, such as a binder, was acting as a nutrient source. Pseudomonads, in particular, have a diverse metabolic activity and can metabolise a wide range of carbon sources including plasticisers (Colbourne, 1979).

Although the silver containing paint was shown to be inhibitory the effects were not maintained over time. The concentration of silver must be maintained within the paint

surface if inhibition is to be preserved, this may result in loss of protection from the non painted surfaces. The paint will need reformulation for use in water systems so that more silver is present and binding agents did not impart nutrient.

CHAPTER 7
DISCUSSION

7.1 AIMS AND OBJECTIVES

The aim of this thesis was to develop a model system so that the ecology of *L. pneumophila* could be investigated. *Legionella pneumophila* is a member of the microbial communities that occur in potable water, hot water systems and cooling towers. Many factors which influence the growth of this pathogen are common to all of these environments and the aim was to produce a model which had relevance in all of these situations. In order for the results of any investigation to be valid, the model system had to be representative of the ecosystem it was intended to model. This was achieved by the use of a continuous culture biofilm model using tap water as the sole source of nutrient and a naturally occurring population of microorganisms which included virulent *L. pneumophila*.

The establishment of a suitable model enabled the investigation of the influence of plumbing materials on biofilm formation and inclusion of *L. pneumophila* in biofilms and investigations of the influence of temperature on the growth of *L. pneumophila* in biofilms. Examination of the three dimensional structure of those biofilms which were developed provided insights into processes which occurred in the biofilm including those involved in the inhibition or supplementation of growth of *L. pneumophila*.

The suitability of several methods of control for *L. pneumophila* was explored using the model system. It was found that biofilms were more resistant to biocide treatments than the planktonic phase.

7.2 BATCH VERSUS CONTINUOUS CULTURE

In order to grow within the environment bacteria must receive nutrients which are utilised and then the waste products of metabolism are excreted. In a closed or batch system the bacterial population increases exponentially in the growth phase and so chemical modification of the environment is also exponential. Modifications in the local environment are due to nutrient depletion, accumulation of metabolic by-products, changes in pH, alteration in oxygen diffusion and shifts in Eh. Although bacteria are capable of responding to changes in the environment and have enormous versatility, in a batch culture growth is inevitably prevented by either insufficient nutrient availability or inhibition by metabolic product accumulation. The lag phase of growth is not due to inherent bacterial properties but a consequence of the changes in the environment.

In the chemostat or continuous culture system media is added and effluent removed at a constant rate resulting in a stable population density within the culture. Growth is most likely limited by depletion of some specific nutrient (the growth limiting factor).

Continuous culture techniques were traditionally used for the production of microbial products (e.g. enzymes or organic acids). Cells can be maintained under optimal conditions for product formation which is at an optimal steady state. The growth rate of the organism is defined by the Monod equation (Monod, 1950) as

$$\mu = \mu_{\max} \cdot \frac{S}{K_s + S}$$

so that changes in the substrate concentration (S) will effect the specific growth rate (μ). Where μ_{\max} is the maximum specific growth rate (h^{-1}), K_s the half rate constant (mg/l) and S the limiting substrate concentration(mg/ml). So according to the Monod equation, growth is limited by a particular substrate and the effect of nutrient changes will depend on the ratio of S/K_s .

The advantage of the use of the chemostat was that cells could be produced constantly and under closely controlled conditions. The environmental conditions could also be varied so that the physiological response of microbes under particular conditions could be studied.

The chemostat has many attributes that also allow useful data to be produced in ecological studies, such as those studied here on the growth of *L. pneumophila* in water systems. The constant removal of effluent and supply of fresh sterile water as medium have many advantages over the batch approach to studying this ecology. The chemostat results in the growth of the organisms within the natural population at a constant rate, rather than the repeated cycle of first exponential growth followed by a lag in growth achieved if batch culture methods are used. Wadowsky *et al.* (1985) used this method for the studying of environmental factors on the growth of the organism which produced ecological data on the ecology of *L. pneumophila*. There were, however, limitations since the cultures have to be continually monitored and the rapid attainment of end points means that the regular reinoculation of sterile water is necessary.

Within chemostat systems toxic metabolic by-products are less likely to accumulate than in batch cultures. Tison *et al.* (1980) used batch culture methods to grow *L. pneumophila* in mixed culture, the accumulation of algal products, however, resulted in elevations in pH which limited the growth of the pathogen. Different results may have been obtained if a continuous culture system had been utilised and this inhibition suggests that the range of conditions in which *L. pneumophila* can grow was

underestimated.

In addition, growth rate conditions in a constantly flowing system follows more closely the events that occur within water systems. In actual water systems, when water is removed for use a fixed volume is maintained by replacement from the mains supply or a holding tank and so for this reason batch systems are less appropriate for ecological studies.

Responses of the bacterial population to environmental shifts can be monitored using continuous culture systems. The responses are directly comparable since all cells are maintained under the same nutritional conditions and under controlled environmental parameters, this results in a reproducible system. Environmental shifts that are of ecological interest and which can be easily controlled in this type of culture system include temperature, pH and oxygen concentration.

The effects of disturbances may also be evaluated using this model system, for example, the addition of either an inhibitory substance or a nutrient by pulsing. These have direct ecological applications such as the modelling of the addition of biocide to a population in a cooling tower.

A number of continuous culture and biofilm models have been previously used to elucidate ecological problems. Multi-stage chemostats have been used to model ecosystems such as the anoxic landfill ecosystems containing methanogens and acetogenic bacteria (Parkes & Senior, 1988). An adaption of this type of system is the gradostat (Wimpenny, 1988) which produced bi-directional gradients by linking five fermenter vessels in series. This type of system has enabled enrichment of different bacterial species but enabling interactions between the organisms within their mutually exclusive habitats. The model described in this thesis is a combination of this type of

continuous modelling combined with biofilm generation (Keevil *et al.*, 1987).

The study of biofilms has included the use of the constant depth fermenter, where biofilm is accumulated in a recess and is suitable for modelling such ecosystems as dental plaque (Wimpenny, 1993). The system has been used to demonstrate the presence of gradients in biofilms of a constant thickness. However, this type of system would be unsuitable for the study of the ecology of *L. pneumophila* since the biofilm is impacted into the recess producing a biofilm which would be artifactual for the modelling of a water system.

An alternative to the holistic approach to the studying of biofilm is the study of attached cells. The physiology of attached cells has been compared with that of newly formed planktonic daughter cells using the methods described by Gilbert *et al.*, (1990). Pure cultures of bacteria were attached to filters, the filters inverted and media flowed through the filters to produce synchronous cultures of attached and planktonic cells. These methods have enabled the comparison of the effect of growth rates and antibiotic activities of attached and free bacteria. Although this method may be useful in elucidating some important components of *L. pneumophila* ecology it was considered first necessary to investigate the behaviour of the microbial consortium.

In an attempt to study the ecology of *L. pneumophila*, the model was inoculated with the whole community of microorganisms present in the calorifier. This type of approach has been used previously in the dental field when Keevil *et al.* (1987) pooled plaque from several patients to inoculate in to a model system. An alternative approach used a defined population of microorganisms obtained from reference collections to study the microbial interactions which occurred in dental plaque (Marsh *et al.*, 1983). It was considered necessary to use the whole population of microorganisms in the model for the study of the ecology of *L. pneumophila* since it was not known which species were important for the growth of the pathogen. In addition, methods are not yet available for

the culture of many aquatic species and it was considered inappropriate to select species on the bases that they grew on laboratory media.

7.3 HOW THE BIOFILM MODEL DEPARTS FROM TRADITIONAL CONTINUOUS CULTURE TECHNIQUES

Most chemostat work has been involved with the growth of pure cultures of microorganisms in defined, nutrient rich media. The model system that has been developed here utilised tap water as the nutrient source where the growth limiting substrate was not known nor could be modified to produce differing growth rates, as an addition of excess nutrient would place unwanted selection pressure on the bacterial population.

The use of a mixed microbial consortium as the inoculum for the model system also resulted in substrate utilisation which has a greater complexity than the Monod calculations anticipate. Although growth rate calculations can be applied to the overall population, individual species must inevitably vary in their growth limiting substrate and optimal growth rate under differing environmental conditions. This is further complicated by the fact that growth rate kinetics are concerned with the aqueous (or bulk) phase of the chemostat since it is assumed that wall growth is an insignificant proportion of the total biomass, consequently growth in biofilms is not included in the calculations.

Interactions that occur within a complex microbial community cannot be accounted for in the calculations either, factors which limit growth of a particular species include competition for nutrient or space, predation and inhibition by other members of the biofilm community. Growth of a particular species may also be enhanced within a community by the local accumulation of growth limiting nutrient by another member of the consortium. It is likely that the overall growth dynamics of the total population may be limited by a particular growth limiting substrate but that a range of metabolic by-

products move along the community structure in a manner unrelated to the growth rate. These metabolic products may serve as nutrients to other members of the community and the metabolite movement will be retarded as biofilm accumulates and the mean generation time may vary vastly from that in the aqueous phase or the cells may remain fixed on the surfaces for long periods of time.

Bacteria and their by-products are engulfed during predation by higher organisms. These protozoa have much longer generation times than bacteria which results in the retention of large quantities of organic material. Until death and decomposition of the protozoa occurs this organic material is unavailable for bacterial growth.

7.4 GROWTH OF *L. PNEUMOPHILA* IN THE CONTINUOUS CULTURE BIOFILM MODEL

Legionella pneumophila is of particular interest in illustrating the inaccuracies of the growth rate calculations. Although the numbers of *L. pneumophila* in the unperturbed first vessel of the chemostat model could be maintained in "steady state" over the three year period with only minor fluctuations to the numbers in the aqueous phase, the factors influencing growth were too complex to calculate.

The biofilm is almost certainly the major site of growth of the *L. pneumophila*. The complex growth requirements of *L. pneumophila* make it unlikely that significant nutrients would be available within the bulk liquid phase to allow growth and division of single cells to reach such an appreciable level so as to contribute to overall increased numbers. The major source of proliferation of *L. pneumophila* in aquatic systems was thought to be within amoebae and protozoa (Rowbotham, 1986). These protozoa are known to accumulate at interfaces so that their grazing is efficient. In the case of engulfment of *L. pneumophila* from surfaces, the bacterial cells may either proliferate intracellularly so as to achieve higher numbers or they may be ingested causing numbers to decline. The outcome of this predation will be determined not only by the species of predator (Rowbotham, 1980) but also by the virulence of the *L. pneumophila* for the protozoa (Wadowsky *et al.*, 1993).

The observation of localised microcolonies of *L. pneumophila* within the biofilm (Section 3.3.6) would suggest that growth may also be achieved extracellularly by means of the supply of nutrients by other microbes within close proximity. Several species of bacteria are known to be able to support the growth of legionellae on media lacking certain nutrients (such as cysteine) and could do so under environmental

conditions. Also present within water environments are bacterial species known to inhibit growth of *L. pneumophila* on agar plates (Rowbotham, 1980, and Toze *et al.*, 1990), these organisms may still be capable of limiting growth within aquatic environments. It is also known that *L. pneumophila* can act as hosts to the endoparasitic *Bdellovibrio* sp. within water systems, which would further reduce their numbers (Richardson, 1990).

Undoubtedly protozoa play an important role in the proliferation of *L. pneumophila* numbers in water systems but there are many other factors that directly or indirectly influence bacterial growth.

It is probable that within some zones of the biofilm, conditions are favourable for the extracellular growth of *L. pneumophila* whilst other regions contain organisms that make conditions unfavourable to growth. Protozoa are known to selectively graze and often show preferences for particular prey. If protozoa show a preference for *L. pneumophila* itself or for bacterial species which supply nutrient to the legionellae, grazing may reduce the *L. pneumophila* numbers by either direct engulfment or by modifying the population to give competitive advantage to bacterial species incapable of supporting or inhibiting the growth of *L. pneumophila*. If the protozoa grazing the biofilm are capable of being hosts to the legionella, predation may result in proliferation of legionella within the protected environment of the protozoa.

7.5 SOME APPLICATIONS OF THE TWO-STAGE BIOFILM MODEL

7.5.1 Ecological studies on the growth of *L. pneumophila*

Despite the problems involved in the mathematical modelling of the complex community in the chemostat model, in practice these "theoretical" considerations were unimportant. The chemostat model maintained both the diverse population over the three year period of this study and the virulence of the *L. pneumophila* as shown in Section 3.3.7.

The usefulness of the chemostat for ecological studies of the aquatic system has been extended by the use of a two-stage biofilm model. The undisturbed first vessel ensured that the second test and biofilm vessel was constantly supplied with a consistent inoculum. This ensured that the flora supplied to the second vessel was reproducible despite modifications in the vessel (such as temperature changes or inclusions of different plumbing materials) and therefore results obtained between experiments were comparable. Man-made systems are also subject to continuous reinoculation from the incoming water and for cooling towers from the air as well, therefore the model represents the situation which occurs in actual systems.

The use of a two-stage model was particularly useful in studying the effect of inhibitory substances, for example, the colonisation of copper and the biocide treatment when there were high levels of toxic material in the vessel at the onset of the experiment. This initial high concentration of inhibitors may have resulted in a loss of diversity which could not have been replaced in a batch system and may have resulted in a suppression of growth of *L. pneumophila* by limitation of the flora required to support the growth of

the pathogen. In the continuous culture system the constant supply of inoculum ensured that diversity was maintained, so that falsely efficient data for the inhibition of *L. pneumophila* by the biocide was not obtained and suppression of legionellae growth was due entirely to biocide action.

7.5.2 Biocide testing

Biocide efficiency is easily assessed against pure cultures of organisms in solutions. This type of work is useful for the preliminary evaluation of minimum dosage against any organism considered important. The usefulness of this type of data is, however, limited. In order to evaluate the efficiency of a biocide it is necessary to test it under conditions similar to those encountered during usage. Biocides intended for use in cooling towers should be tested within actual cooling towers to ensure efficiency and suitability before release for general use. These cooling tower trials could take place immediately following the preliminary dosing experiments, however, there are several reasons for using a laboratory based trial prior to this stage. One economic and ethical reason for more extensive investigations prior to actual cooling tower trials, is that the biocide may be ineffective with possible release of contaminated aerosol from the cooling tower. The use of a model system to determine more precisely the required dosing requirement for a cooling tower trial may reduce actual fieldwork costs and provide a contained method for this evaluation.

Cooling towers are large structures and the representative sampling of the flora within them is difficult to achieve, this is often attempted by removal of planktonic samples only. A wide range of environmental gradients exist within cooling towers and the opportunities for the growth of *L. pneumophila* vary accordingly. It is possible that the legionellae survive and grow within an area that is overlooked by the sampling methods

involved. The internal and external conditions in cooling towers vary with season or usage, therefore long term experiments or comparisons of treatment regimes may be completed under differing environmental conditions and consequently may not have comparable results. The cooling tower cannot be operated "worst-case conditions" where high numbers of *L. pneumophila* can be maintained within the system because this would pose unacceptable health risks to both workers and the local community.

The chemostat model has been developed to act as a suitable environment for testing biocides against both planktonic and more resistant biofilms simultaneously. Biofilm and planktonic effects can be easily and representatively sampled to determine biocide efficacy. The chemostat can be operated under "worst case conditions" to ensure high numbers of *L. pneumophila* for the production of biofilm and testing of biocide by the constant supply of inoculum from the unperturbed first vessel. The biocide can be tested against preformed biofilm or against the establishment of biofilm on clean surfaces during constant biocide addition.

7.6 DISCUSSION OF RESULTS OBTAINED FOM THE MODEL SYSTEM

7.6.1 Colonisation of glass surfaces

The experiments described in Section 3 were concerned with the development of the model system and the investigation of the colonisation of glass surfaces. Reproducibility of biofilm development was determined, three-dimensional structures of biofilms was analysed and the relevance of the model to the aquatic environment was ensured since the virulence of the *L. pneumophila* was shown to be conserved. In particular, the use of a two-stage biofilm model was significant. It allowed modification of the environment where biofilms were produced without loss of bacterial diversity or alterations in the populations of bacteria in the culture. Therefore, throughout the investigations, the composition of the aquatic consortia reflected those of the original calorifier responsible for the outbreak so that the investigations were carried out using a consistent population and results could be directly compared between experiments.

The microbial species present in the planktonic and biofilm phases were predominantly Gram negative bacteria, many of which have previously described as being present in water systems (Olsen and Nagy, 1984) and this is clearly essential since any model system should maintain a microbial community which resembles the ecosystem that it intends to model (Gottschal and Dijkhuizen, 1991).

The use of sterile tap water as the sole source of nutrient and the use of a naturally occurring inoculum were undoubtedly critical in ensuring that a suitable community of microorganisms was maintained. Amoebae and other protozoa were maintained as community members, some of which were known to be hosts for *L. pneumophila* (Wadowsky and Yee, 1985). The presence of amoebae and other protozoa was considered essential, especially for experimentation where biocide activity was to be

assessed since these organisms are known to protect the pathogen from biocide treatments (Kilvington and Price, 1990). The presence of amoebae in uncontaminated drinking water has been recognised for many years (Chang, 1960) and these protozoa are present on the surfaces in water systems (Dott *et al.*, 1979). Such protozoa form a normal part of the aquatic community in water systems and therefore it is important that they constitute a component of consortia in the model system.

A considerable part of Section 3 was concerned with the three-dimensional structure of the biofilms which were developed on the surface of the glass tiles. Information provided by the use of light microscopy, scanning electron microscopy and scanning densitometry combined to provide insights into biofilm topography. All of the methods showed that biofilms were mosaics in terms of total biofouling with some regions being relatively uncolonised whilst dense patches of biofilm formed in localised regions. This type of structure has been observed in actual water systems (Pederson, 1990) and the structure conforms to that described by Characklis and Marshall (1990). Biofilms formed on the surface of a variety of plumbing materials and under different temperatures were found to have similar structures when examined using the scanning electron microscope (Sections 4 & 5). The biofilms which formed varied in thickness between 5-100 μm in depth which is within the range of depths previously reported for biofilms produced under a variety of conditions (Christensen and Characklis, 1990).

The observations of striated bacterial microcolonies (Figure 3.6) with clusters of cells of similar morphologies (Figure 4.22) would indicate that growth was occurring within the bacterial community and indicated that co-operation was occurring between bacterial species. The immunolabelling of *L. pneumophila* (Figure 3.8) within the biofilm community indicated that the pathogen could grow outside of protozoa presumably with nutrient being gained extracellularly from the microbial consortia. A similar staining procedure used previously by Schofield and Locci (1985) has also demonstrated clusters of *L. pneumophila* in biofilms in the absence of significant numbers of protozoa.

7.6.2 The effect of material selection of growth of *L. pneumophila*

After establishing the suitability of the model system in section 3, experimental work described in section 4 went on to determine the impact of plumbing material selection on total biofouling and growth of *L. pneumophila*. The materials were shown to vary in their ability to form biofilms and support *L. pneumophila* as a part of the mixed microbial consortia, despite the fact that they were exposed to the same microbial community under identical environmental conditions. The data supports observations made by Schofield and Wright (1984) and Schofield and Locci (1985) but data presented here provides additional information since the materials were colonised independently and the progression of biofilm formation was examined by enumeration of *L. pneumophila* and total populations. The model described by Schofield and Locci (1985) was a closed system with intermittent water recirculation where strips of the materials were packed into tubes for colonisation. Bacterial colonisation was examined by immunofluorescence only and no statistical examination was possible.

Material selection has recently been shown to effect the colonisation of *L. pneumophila* in a rig system using immunofluorescence to determine the numbers of *L. pneumophila* on the material surfaces over 7 days (Wreiman *et al.*, 1993). Statistical analysis was not undertaken and the authors did not assess the viability of the *L. pneumophila* or enumerate the non-legionella populations.

Data presented here contradicts the results of Wright *et al.* (1989) who found material selection had no effect on the colonisation of *L. pneumophila*. The differences in results can be explained by differences in methodology since Wright *et al.* (1989) used pure cultures of *L. pneumophila* grown in 10% algal extract for the colonisation of douglas fir, carbon steel, galvanised steel and copper.

No difference was found in the colonisation of different materials by *L. bozemanii*, with a maximum of 7.0×10^4 cfu cm⁻² (Green and Pirrie, 1993). The differences in results presented in this thesis and the results of these authors is possibly due to differences in the metabolic activities of *L. bozemanii* and *L. pneumophila*. However, the authors used a one stage model and commented that some inhibitory substances were present which may have resulted in reduced bacterial diversity and may explain the lower numbers of legionella on the surfaces. In addition, no amoebae were present and *L. bozemanii* was added to the inoculum after growth on solid media, rather than being included as a part of a naturally occurring population of microorganisms.

Results presented in Section 4.3.9 indicated that the high numbers of *L. pneumophila* and other bacterial species colonising the surface of elastomeric materials was, at least in part, due to increases in available organic carbon released from the materials.

The influence of materials selection on the growth of *L. pneumophila* was shown to be maintained over a wide temperature range (Section 5). Copper was consistently less colonised than the plastics tested at 20, 40, 50 and 60°C and the numbers of *L. pneumophila* was significantly lower on the copper surface. Copper was found to inhibit the colonisation of the glass control tiles suspended in its presence and of the planktonic phase (Table 5.1), inferring that the use of copper pipe may also protect other minor components, eg. washers, from colonisation. Importantly, the levels of copper ions found in the aqueous phase of the model system were maintained below the recommended levels for copper in drinking water for the duration of the experiments (Section 5.3.7), which was considered essential for the suitability of the model in predicting the impact of copper leaching.

7.6.3 Effect of temperature on the growth of *L. pneumophila*

The impact of temperature on biofilm formation and growth of *L. pneumophila* in biofilms was presented in section 5. *Legionella pneumophila* was shown to be only a minor component of the biofilm flora on plastic surfaces at 20°C and 50°C but absent from copper surface at these temperatures. At 40°C *L. pneumophila* was most abundant, particularly on the plastic surfaces where populations of the pathogen accounted for half the biofilm flora. At 60°C the *L. pneumophila* could not be detected in the model system.

There are several implications for the control of *L. pneumophila* which can be inferred from this work. The presence of *L. pneumophila* in biofilms at 50°C is of concern, since it would indicate that systems operating at this temperature may well contain low numbers of the pathogen along with a supporting population of microorganisms within biofilms. Although this may not be of concern if system temperatures can always be maintained, it may be of greater importance if temperatures were to fall by only a few degrees, the pathogen may then rapidly multiply on the biofilm surface to high numbers of which might possibly lead to infections of humans.

Many water systems are operated at lower water temperatures than those suggested by the DHSS guidelines (Anon, 1989) because of energy costs, concern over patient scalding or the inability to attain the required temperatures in older systems. The use of copper pipe would possibly be beneficial in minimising the risk of Legionnaires' disease, particularly if systems were operated at lower temperatures than those recommended in current guidelines.

The presence of *L. pneumophila* at 50°C is unlikely to be due to growth at this temperature since the decimal reduction time of *L. pneumophila* is less than 2h at this temperature (Dennis *et al.*, 1984). The possibility that amoebae can protect *L.*

pneumophila from high temperatures has already be shown to be unlikely (States *et al.*, 1993). The data would indicate that numbers of *L. pneumophila* were in some way protected by the biofilm since numbers slowly accumulated over time.

7.6.4 Extracellular multiplication of *L. pneumophila* within the biofilm community

It is undoubtedly true that *L. pneumophila* are capable of growth within amocbae and other protozoa and this is an important mechanism for the proliferation of legionellae in the aquatic environment. Intracellular growth has been suggested as the sole mechanism for replication in the environment by several workers, *eg.* Rowbotham (1980). There are several competitive advantages to be gained by the intracellular mode of growth of legionellae and these include escape from other predators, enhanced nutritional status in the absence of competing organisms and protection from biocide treatments.

The organism survives in a diverse range of habitats which would suggest that hosts are not necessarily essential for the growth of the pathogen. Rowbotham (1980) reported the presence of legionellae in the absence of detectable amoebae within hot water systems suggesting that this may be the case. It has been stated but not proven that legionellae are obligate parasites of protozoa. In pure culture studies *L. pneumophila* are capable of growth extracellularly in a defined medium (Mauchline and Keevil, 1991) and it would seem advantageous for them to exploit this mode of habit in favourable conditions in the aquatic environment.

Wadowsky *et al.*, (1988) attempted to determine factors which are most important for the growth of legionellae in tap water. It was shown that *Hartmanella sp.* were capable of supporting growth of *L. pneumophila* and that some filterable factor of 2 - 5 μm in

size was essential for the growth of legionellae under the conditions that were tested. This data would appear to support the view that protozoa are essential for the growth of legionellae, however, the batch type system used may have been unfavourable and so excluded the growth of some essential bacterial genera required by the legionellae for extracellular growth. It is possible that alternative growth temperatures or water conditions may have been more conducive to bacterially enhanced growth rather than intracellular growth but these were not included in the experiments.

The data presented in this thesis suggests that other mechanisms may be important for the proliferation of legionellae in the aquatic environment. The amoebae that could be detected in both the planktonic and biofilm phases at 20 °C were no longer detectable at 40 °C in the chemostat model (Section 5.3.3). This decrease in host numbers coincided with elevations in the numbers of *L. pneumophila*. It could be that the amoebae were no longer able to grow within the system due to unfavourable temperature conditions resulting in depletion in numbers below the detection limit. If amoebae were absent then the legionellae must have achieved high growth rates in alternative sites. The other possibility is that the numbers of protozoa was reduced by rapid intracellular proliferation of the legionellae resulting in lysis of the protozoa and enhancement in the numbers of legionellae.

The data presented here indicates that biofilm is capable of supporting the extracellular growth of the *L. pneumophila* because immunolabelled biofilm contained discrete microcolonies (Section 3.3.6). These cells were all short rods implying they were growing rapidly, presumably due to the nutrient supplied by other bacterial members of the biofilm population. The other factor which suggested *L. pneumophila* is capable of an extracellular mode of growth was the absence of amoebal cysts. There are several reports of the failure of legionellae to infect cysts. Since cyst formation is not uncommon in water, detection of some cysts would be expected even if free living, but actively growing amoebae were then lysed following parasitisation.

If amoebae were the sole mechanism for the growth of legionellae in the model system, then some indication of the numbers required to sustain the legionellae population can be calculated. Although the estimation of the numbers of *L. pneumophila* present within infected protozoa vary enormously, estimations are of use for approximation of turn over rate. According to estimations by Rowbotham (1984) *A. polyphaga* contained between 326 and 1,320 *L. pneumophila* but by 1986 the author concluded that large amoebae were able to accommodate as many as 10,000 *L. pneumophila*. These estimations of intracellular replication by *L. pneumophila* greatly exceeded the numbers estimated by Vandenesch *et al.* (1990) who counted 10 - 100 bacteria present within cytoplasmic vacuoles within infected amoebae.

At the maximum growth rate that occurred within the chemostat model the planktonic phase maintained a legionella population of around 5×10^3 cfu ml⁻¹ in a 500 ml volume so that the planktonic population contained 2.5×10^5 cfu legionellae. The glass surface supported biofilm containing between 1×10^4 and 4×10^5 cfu cm² of legionellae of non inhibitory surfaces. The surface area of the vessel was approximately 500 cm² so biofilm flora contained approximate legionellae numbers of between 5×10^6 and 2×10^8 . This would suggest that the total legionellae population in the vessel was between 5.3×10^6 and 2×10^8 .

Since the dilution rate of the vessel was 0.2 h⁻¹ with inoculum entering the vessel at a dilution rate of 0.05 h⁻¹, the vessel received nutrients at a rate of 75 ml h⁻¹. Therefore, the actual dilution rate of fresh medium would be 0.15 h⁻¹ and the mean generation time would be 6 h 40 min. If each amoeba contained 326 bacterial cells the total number of amoebae required to maintain the numbers of legionellae within the model system would be between 1.63×10^4 and 6.14×10^5 , depending on which of the two total legionellae populations estimations are used. If amoebae contain 1320 legionellae the number of amoebae required would be 4.02×10^3 to 1.5×10^5 , if the amoebae contain

10,000 legionellae cells the number of amoebae required would be 5.30×10^2 to 2.0×10^4 , if amoebae contained only 10 legionellae the estimated amoebae population would need to be 5.3×10^5 to 2.0×10^7 and if amoebae contained 100 bacterial cells then the amoebal population would be 5.3×10^4 to 2.0×10^6 .

These estimations are the numbers of amoebae required to maintain the numbers of *L. pneumophila* in the model system over a generation time of 6 h 40 min. These estimates assume all the amoebae present are colonised with legionellae. However, in order to maintain the population of legionella the total amoebal population would have to be higher in order to replace amoebae killed by parasitising legionellae. The estimations err on the side of caution because the calculation assume infected amoebae numbers only and no estimation of the number of non infected cysts was attempted. These estimations are based on the numbers of *L. pneumophila* capable of infecting *Acanthamoeba* sp., the model system did not contain such large amoebae and so numbers of amoebae required to act as hosts is further underestimated.

Although these estimations are extremely crude and based on many assumptions, the data serves to show that for most of the estimations of amoebal capacity, detection of the amoebae should be possible. The fact that no amoebae were apparently present would suggest that the carrying capacity of the model ecosystem studied could not be maintained by the intracellular replication within protozoa.

The argument against obligate parasitism by *L. pneumophila* is further supported by the observations that populations of *L. pneumophila* which are avirulent to man are present within the environment. If avirulence in humans is related to avirulence in amoebae, then avirulent legionellae capable of surviving in the aquatic environment must replicate extracellularly. If these bacteria were engulfed then the protozoa would ingest them, and cause selection pressure to reduce numbers. Thus, in the absence of an alternative mechanism for replication, the population of legionellae would decline until extinction.

Since non infective populations of *L. pneumophila* persist in the aquatic environment it would imply that other mechanism are present to enable growth of not just avirulent but also virulent populations of the pathogen.

7.6.5 A hypothesis on the development of aquatic biofilms.

The biofilms which form on the surface of plumbing materials are influenced by the temperature of the environment. Those bacteria capable of surviving or growing within a particular operating temperature range are capable of attaching to the surfaces. The nature of the plumbing materials will influence the extent, speciation and diversity of microorganisms which are primary colonisers. In the case of latex and ethylene-propylene which have been shown to be nutrient supplying surfaces those bacteria (or consortia of bacteria) which are capable of utilising the nutrient as substrates will move to the surface, possibly by chemotaxis, and will attach to form biofilms.

Plastic surfaces which do not promote growth by direct nutrient supply may offer competitive advantages to bacterial species by permitting secure attachment to the substrate so that these cells can gain indirect nutritional advantages. This may be due to protection from shear forces in crevices, to the hydrophobic/hydrophilic nature of the substrate or to the surface charge of the materials.

Some surfaces will be inhibitory to colonisation of many bacterial species due to the hostile nature of the material, as is the case for copper and silver paint. Those few species of microorganisms capable of tolerating the high levels of copper or silver ions would be free to colonise the surface in the absence of other competing microorganisms.

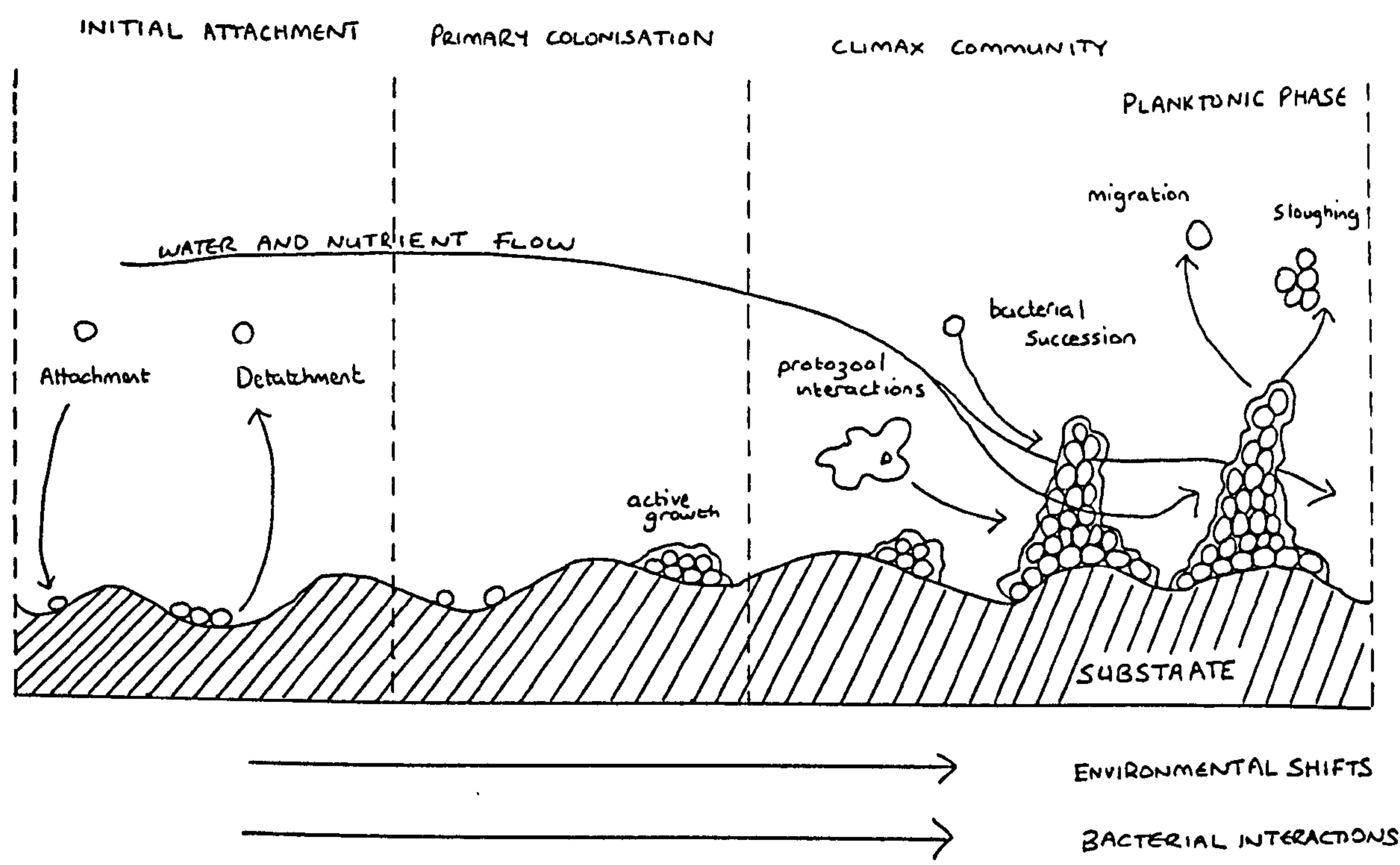
As the selection of plumbing materials influences the primary colonising species, this

will in turn influence the bacterial succession which will take place. Those bacteria which colonise the surface will either remain dormant under unfavourable conditions or will grow to form microcolonies under more suitable conditions. The growth of these pioneering microorganisms will inevitably lead to modifications of the immediate environment surrounding the cells by the reduction in any available nutrient from the supporting surface and by the accumulation of metabolic by-products. These metabolic by-products may be metabolites for other species, may be toxic to other species or may modify the pH of the local environment.

In the case of bacterial succession on copper, some areas of the naked surface of the material will be covered by localised regions of biofilm where pioneering species and there extracellular material has accumulated. In addition calcium carbonate, humic and fulvic acids or other chemical components of the aqueous phase may form passivating films over the copper surface. These films and regions of biofilm may be more favourable for the colonisation of less copper tolerant species, possibly by the spatial separation of succeeding bacterial species from the copper surface.

Thus the modifications of the local environment by the pioneering microorganisms within the biofilm will lead to bacterial succession which will result in an eventual climax community of microorganisms. Some dormant cells or newly attached cells will find conditions favourable for growth whereas some pioneering species will suffer from intraspecific competition and will have sufficiently modified their local environment to find less favourable conditions and will succumb to interspecific competition.

Figure 7.1 Schematic diagram of the factors involved in biofilm development.



Bacterial cells will be removed from the biofilm by three major mechanisms grazing, sloughing and migration. Grazing by protozoal populations may selectively reduce numbers of some bacterial species depending on the feeding preferences of the protozoa present. Although *L. pneumophila* may be capable of intracellular replication within protozoa, most grazing activities will result in a reduction in bacterial populations and will result in the absorbed nutrients being unavailable to the other bacteria for the generation time of the grazing microorganisms.

Sloughing of bacterial cells occurs when biofilm microorganisms are lost to the planktonic phase by an involuntary process. This may occur when bacteria close to the material surface cannot attain sufficient nutrient or oxygen and die, releasing clumps of materials into the aqueous phase as cell adhesion is lost. Alternatively, cells may be lost by shear forces or by local turbulence caused by the grazing activities of protozoa.

Active migration may occur when conditions become unfavourable due directly to accumulation of toxic metabolic by-products or indirect pH modification. Cells may also migrate if diffusion of nutrients or oxygen through the microcolonies becomes insufficient to sustain growth as inter and intraspecific competition increases.

7.6.5 Three dimensional structure of aquatic biofilms

The biofilms which developed on the surface of the different plumbing materials had several common features. Microcolonies of bacterial cells of similar morphology could be observed on most surfaces and this would indicate that growth of microorganisms was following a mode of growth similar to that occurring on solid agar.

These mosaics of microcolonies of bacteria were also indicated by the presence of small

areas of different polymer types, indicating that the local environment within the biofilms was modified by primary colonising species (Section 5.3.8)

Most surfaces, with the exception of the elastomeric surfaces, did not form dense biofilms which completely covered the surface. More commonly, the materials had small, localised regions of biofilm which were interdispersed by uncolonised regions. The proportion of these biofouled regions increased over time, but in most cases, dense mats of biofilm did not form. There are several implications of these results since assumptions have been previously made based on the biofilm being a dense mat of microorganisms of uniform thickness.

The structure of biofilms observed by light microscopy, scanning electron microscopy and demonstrated by scanning densitometry would suggest that an open structure exists in biofilms. This type of biofilm structure would seem more suited to bacterial growth and succession. A dense mat of microorganisms would lead to problems of gaseous diffusion, nutrient availability and release of metabolic by-products. It would also seem probably that cells would not just accumulate but cells would also migrate from the surface of biofilm as conditions become unfavourable. The proposed more open structure of biofilms would seem more plausible for aquatic biofilms where conditions remain aerobic, where nutrient availability is always low and migration could then occur. In addition, protozoa also produce turbulent eddies during grazing activities and movement within the biofilm may form channels within the biofilm structure by the physical displacement of bacteria.

Although this structure is proposed for the type of aquatic systems where *L. pneumophila* occurs, further work is necessary to determine if this type of structure occurs in other ecosystems, eg. in dental plaque in the mouth or in oil pipelines.

7.6.7 A hypothesis on the inclusion on *L. pneumophila* in biofilms

The biofilms which develop on the surface of the different plumbing materials have been shown to vary in ability to support the growth of *L. pneumophila*. Those factors which influence speciation and succession of the biofilm population will also influence the numbers of *L. pneumophila*.

Bacterial species present in the aquatic environment will vary in their ability to support the *L. pneumophila* population. Some species of bacteria are known to be able to supplement *L. pneumophila* with cysteine, an essential growth requirement, on solid media (Wadowsky and Yee, 1982). Microorganisms capable of supporting growth include cyanobacteria (Tison *et al.*, 1983), *Pseudomonas* spp, *Flavobacterium* spp., *Alcaligenes* spp. and *Acinetobacter* spp. (Stout *et al.*, 1985). Although cyanobacteria are unlikely to play a major role under the dark conditions that exist in most water systems, it is possible that these microorganisms may enter water systems and that their death and decomposition may lead to release of useful nutrients for the extracellular growth of the pathogen. It would appear more likely that the actively growing members of the bacterial community supply the major source of nutrient for the extracellular growth of the *L. pneumophila*. The populations of microorganisms included in the chemostat model system included members of those species described by Stout *et al.* (1985) as being able to supplement the growth of the pathogen. Since microcolonies of *L. pneumophila* could be viewed within biofilm populations using immunolabelling techniques, it would appear likely that several species of bacteria present within the biofilm communities will be capable of supporting extracellular growth of *L. pneumophila*.

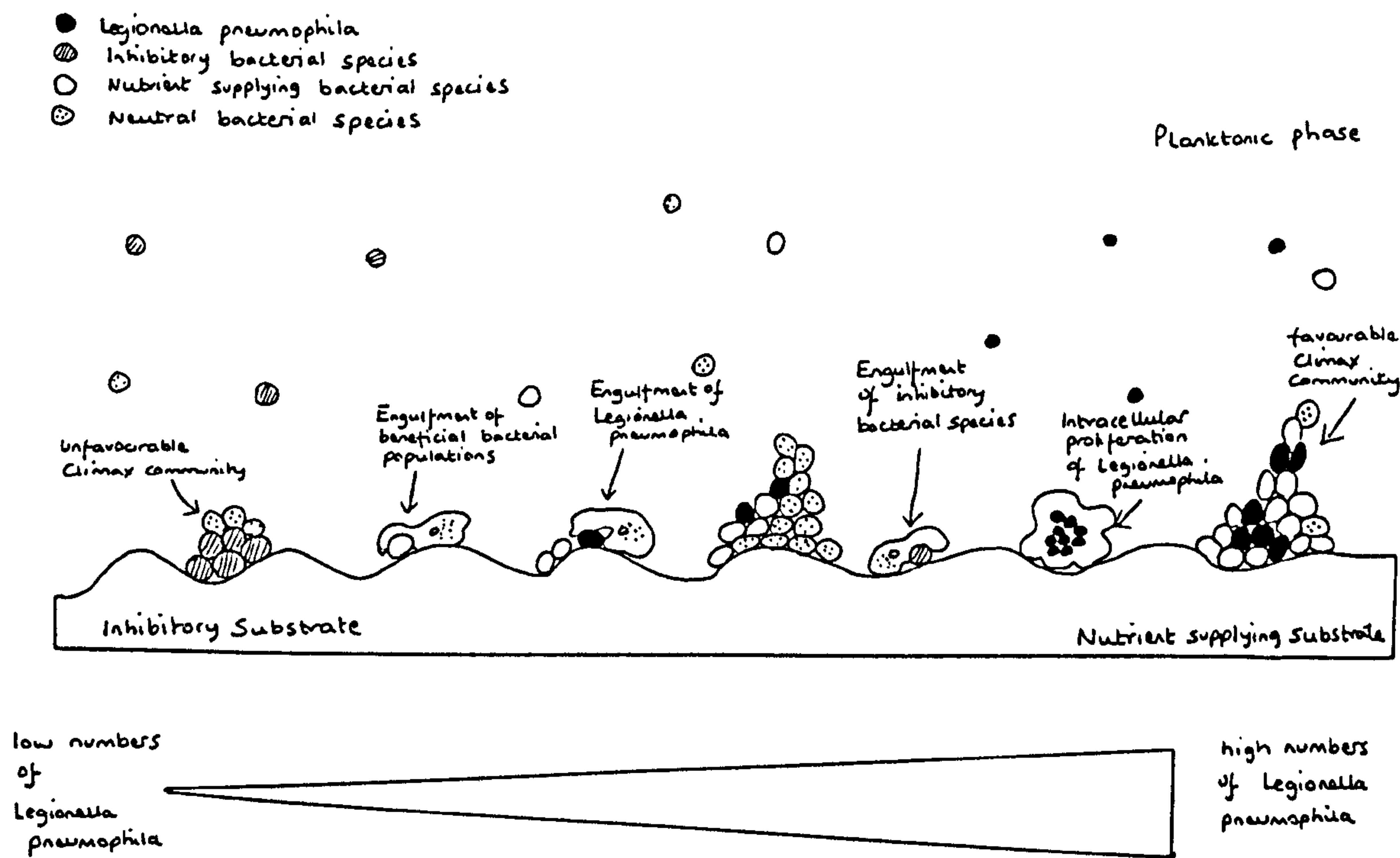
Some members of the microbial community are known to produce chemical compounds which are inhibitory to other bacterial species. One important member of the microbial

community which is known to inhibit *L. pneumophila*, is *Pseudomonas aeruginosa* because purified pyocin from this microorganism is sufficient to inhibit growth of *L. pneumophila* on agar plates (Rowbotham, 1980). *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Aeromonas* spp. are known to produce bacteriocins which inhibit *L. pneumophila* on agar plates (Gomez-Lus *et al.*, 1993) and *Klebsiella* spp. and *Aeromonas* spp. were also present within the model system. Although it could be argued that the presence of these microorganisms within the biofilm populations has not been shown to inhibit the growth of the *L. pneumophila in situ*, it is likely that these microorganisms would produce these bacteriocins in the natural environment, where a definite competitive advantage could be gained.

Other community members may have no direct effect on the growth of the pathogen and their actions could be termed neutral. However, these microorganisms could be major primary colonisers and could possibly produce those conditions which influence bacterial succession and thereby indirectly influence the *L. pneumophila* populations. If the climax community was predominantly composed of these microorganisms then *L. pneumophila* would be neither discouraged or augmented by the biofilm flora.

Biofilms which supported high numbers of bacterial species capable of supplementing the growth of *L. pneumophila* would be expected to contain high numbers of the pathogen. If the climax community was dominated by microorganisms which produce unfavourable conditions for the growth of *L. pneumophila* then the pathogen would be present as a low proportion of the total microbial flora. Copper surfaces supported sparse biofilms but *Pseudomonas aeruginosa* was a component of the tolerant population, this would indicate that copper toxicity was possibly not the only mechanism inhibiting the numbers of *L. pneumophila* in the biofilms forming on the copper surface. Immunolabelling showed that the *L. pneumophila* was not underestimated by recovery on culture media and this would suggest that the *Pseudomonas aeruginosa* was not just inhibiting recovery on agar media.

Figure 7.2 Schematic diagram of the factors which influence the number of *L. pneumophila* present in biofilms



In activated sludge, protozoan grazing has been shown to be a highly selective process, leading to the dominance of certain bacterial types (Gude, 1979) Protozoal populations undoubtedly have a role in the shaping of the climax community of the biofilm. Selective grazing of bacterial populations may lead to modifications of the resulting bacterial populations. If grazing pressures reduce those bacterial species capable of sustaining *L. pneumophila* within the biofilm community, then numbers of the pathogen may decline. In contrast, if those species which are antagonistic to the growth of the pathogen are reduced by engulfment by protozoa, then the *L. pneumophila* numbers may increase. In addition to the indirect influence of protozoal populations on *L. pneumophila* populations by the modification of the populations of other bacterial species, *L. pneumophila* populations could be influenced directly. *Legionella pneumophila* can proliferate by intracellular replication within several species of amoebae however, some species of amoebae do not support intracellular replication of the pathogen and engulfment results in absorption. Therefore engulfment of *L. pneumophila* by amoebae could lead to enhancement or reductions in the numbers of pathogen possibly depending on the virulence of the bacteria and the particular protozoa encountered.

There were several trophic levels present in the model ecosystem and these microorganisms would undoubtedly interact to influence populations of microbial species. The amoebal species present within the model system would have been suitable prey for the rotifers which were also present. Rotifers are known to feed on amoebae and these interactions will in turn influence *L. pneumophila* numbers (Rowbotham, 1980).

7.6.8 Testing the suitability of materials for use in water systems

The current British Standard (BS 6920) is concerned with the overall quality of potable water and is not concerned with effects of plumbing material on particular microorganisms. Materials used in potable water have been demonstrated to have an impact on the growth of *L. pneumophila* (Section 4 and 5) and it could be argued that the BS 6920 should be modified to include the effect of material selection on the growth of this pathogen. This thesis has, however, been concerned with the growth of *L. pneumophila* not just in potable water supplies, but also in hot water systems and cooling towers. In addition, water systems operating at the low temperatures (less than 20°C) which occur in cold potable waters did not support high numbers of the *L. pneumophila* (Section 5). *Legionella pneumophila* was most prolific at 40°C which is well above the temperatures found in cold water systems, but may occur in hot water systems and cooling towers. If BS 6920 was modified to include *L. pneumophila* the systems where *L. pneumophila* was of greatest concern would not be included.

The introduction of suitability testing for materials in use in hot water systems and cooling towers may be of some value, since guidelines could then be produced for environments at risk from the growth of *L. pneumophila*, eg hospital hot water systems and cooling towers. The guidelines should include places where there are large numbers of susceptible people, such as hospitals or old peoples homes, particularly where larger and less controllable water systems are operated. Water systems in domestic use generate only a minimal health risk from growth of *L. pneumophila* (Alary and Joly, 1991) since these systems are small and temperatures can be more closely controlled, therefore it would be unnecessary to include these systems in extra testing procedures for material suitability.

7.7 CHEMICAL CONTROL OF *L. PNEUMOPHILA*.

The suitability of a range of commercially available biocides, at the suggested recommended doses, were tested against *L. pneumophila*. The two-stage model system enabled biocide efficiency to be compared for the biofilms and planktonic *L. pneumophila* (Table 6.1).

The non-oxidising algicide, GCO-30, was ineffective against both planktonic and sessile populations of microorganisms and *L. pneumophila* even at doses higher than those suggested for use in cooling towers. Although another non-oxidising algicide, WCO-130-30E, was found to kill bacteria in the planktonic phase, the biocide was less efficient against the biofilm populations. *Legionella pneumophila* was however, removed by this biocide but excessively high concentrations and contact times were required.

Dubact B, an oxidising biocide, was effective in reducing *L. pneumophila* and other microorganisms in both biofilm and planktonic phases, however, the biofilm populations of *L. pneumophila* were not completely removed by the biocide treatments. Although bromo-chloro, dimethylhydantoin has previously been shown to be effective against *L. pneumophila* (Kurtz and Davies, 1988) in actual cooling tower trials the biocide was ineffective at controlling *L. pneumophila* (Fliermans *et al.*, 1984). Data presented in this thesis would suggest that failure of disinfection was due to the survival of *L. pneumophila* within biofilms during the biocide treatment. Biocides have been previously shown to be less effective against mixed biofilm populations containing *L. bozemanii* (Green, 1993) and against attached *L. pneumophila* in pure cultures (Wright *et al.*, 1991).

The three dimensional structure of biofilms that was proposed in Section 7.6.5 has an open structure where channels are present in the biofilm which allow nutrient and water flow. The results presented in table 6.1 demonstrate that biocide activity is reduced in biofilms when compared to the planktonic phase. Initial observations would suggest that the more open biofilm structure would enable biocides to easily gain access to bacteria in biofilms via these channels. The apparent contradiction can be explained by the observation of microcolonies of bacteria within biofilms, biocide must penetrate these before inactivation of the bacteria at the centre of the microcolonies can take place. Although these microcolonies may be considered a smaller barrier to the penetration of biocide than the dense layers of biofilm previously envisaged, they will still require high doses of biocide since biocide will be absorbed and microorganisms will be protected.

Silver paint was demonstrated to inhibit the initial colonisation of surfaces (Section 6.3.2.2) and prevent growth of *L. pneumophila* (Section 6.3.2.3). Although the inhibition was not sustained for more than two weeks, probably due to the gradual loss of silver ions from the paint surface, it would indicate that the use of silver ions may be suitable for the control of *L. pneumophila* within mixed populations within water systems. Pure cultures of *L. pneumophila* in filtered water have been shown previously to be susceptible to electrolytically generated copper and silver ions at a concentration of 40 µg/l in the presence of 0.4 µg/l free chlorine (Landeem *et al.*, 1989). The model system described here could be useful in enabling the activity of this type of silver/copper ions generating devices to be tested against mixed communities of microorganisms and against biofilm populations.

The model system has been useful for the comparison of different methods of control of *L. pneumophila* and other biofouling microorganisms. The use of the two-stage model is particularly useful since it provides a constant challenge of microorganisms for which

biocide activity is to be assessed for the duration of the experiment and ensures that the diversity of the microbial population is maintained.

Several biocides may be simultaneously tested if colonised plumbing materials were removed from the model system for external assessment (as in Section 6.3.1). If testing of continuous biocide efficacy was required then biocide activity could be investigated in the model system (as in Section 6.3.2.2). A modification of the model system would be suitable for determining the continuous activity of several biocides simultaneously. The seed vessel could be used to provide a constant inoculum to several biofilm generating vessels arranged in parallel and the biocide efficiencies of several different biocides could be simultaneously examined.

7.8 CONCLUSIONS

1. The model system described in this thesis produces a suitable method for examination of the ecology of *L. pneumophila*.
2. The presence of *L. pneumophila* in microcolonies in biofilms, in the absence of protozoa, would indicate that the *L. pneumophila* was growing extracellularly by receiving nutrients from the bacterial consortium. Therefore *L. pneumophila* should be considered to be a facultative intracellular parasite.
3. Growth of microorganisms in biofilms is influenced by plumbing material selection, with copper inhibiting colonisation and elastomeric materials providing nutrients for growth.
4. Growth of *L. pneumophila* in biofilms is influenced by the plumbing material, with copper being inhibitory, elastomeric materials supporting high numbers and plastic materials being intermediate. Testing plumbing materials and selecting those which discourage growth of *L. pneumophila* may be one method for controlling numbers in areas where their growth may lead to infection (eg. hospital water systems and cooling towers).
5. The environmental temperature has a dramatic effect on the growth of *L. pneumophila* in biofilms. Growth at 20°C is poor, but at 40°C up to 50% of biofilm flora may be *L. pneumophila*.
6. *Legionella pneumophila* is still present in biofilms which develop on plastic materials at 50°C, indicating that the biofilm is capable of protecting the bacteria from

the effect of heat. This would suggest that water systems operating at 50°C may contain viable *L. pneumophila*, along with associated microflora, and therefore a relatively small reduction in the operating temperature could rapidly result in high numbers of the pathogen.

7. No viable *L. pneumophila* could be found in the biofilm or planktonic phases of the model system operating at 60°C. This would indicate that by operating water systems at this temperature the risk of infection would be removed.

8. Some commercially available biocides used for controlling algae in cooling towers were not suitable for controlling *L. pneumophila*. This would imply that biocides which are found to control biofouling in cooling towers may not control *L. pneumophila* populations and therefore the biocide should be specifically tested for suitability in controlling *L. pneumophila*.

9. Biofilms containing *L. pneumophila* were resistant to biocides which were successful in controlling planktonic *L. pneumophila*. The testing of biocides in this type of model system on biofilm and planktonic populations of *L. pneumophila* may provide more accurate information of the dose requirements and predicted biocide activity that would occur in actual cooling towers.

7.9 FUTURE WORK

The model system has provided a useful holistic approach to studying the ecology of *L. pneumophila* and has provided important information on the impact of temperature and material selection of the growth of *L. pneumophila* in biofilms. This work could be further extended by examining the impact of intermediate temperatures, particularly between 30-50°C. Since virulence of *L. pneumophila* has been demonstrated to be influenced by temperature (Mauchline *et al.*, 1993) it would be of particular importance to determine if the highest numbers of the pathogen coincided with highest virulence. The model system could also be used to investigate the role of other environmental conditions, such as water velocity on the general biofilm formation and how this influences colonisation by *L. pneumophila*.

Legionella pneumophila is the most commonly isolated *Legionella* spp. in water systems and the most common cause of Legionnaires' disease. The model system could be used to determine the interactions which occur within legionella populations which lead to the prevalence of *L. pneumophila* by including a mixture of different legionella species. Further work could also be obtained concerning the virulence of the different species grown under different environmental conditions.

Further work is required on the structure and interactions which occur within biofilms. Biofilms could be generated in the model system and then examined using a diverse range of different procedures. Activities could be examined *in situ* by examining the biofilms using metabolic dyes, radioactive tracers, or using species specific bacteriophages. These may provide different approaches for evaluation biofilm activity or biocide efficiency.

It would be interesting to resolve those factors which influence the hypothesis presented on biofilm formation and inclusion of *L. pneumophila* (section 7.6.5 & 7.6.7) and this would require a less holistic approach. The predator/prey interactions which occur within the biofilm community could be examined to determine which species of protozoa have particular prey preferences. This may also be a suitable method for determining the predation/parasitisation interactions which occur between *L. pneumophila* and the protozoal community.

The bacterial interactions which occur between *L. pneumophila* and the other biofilm bacteria would be interesting to unravel. By isolation of the bacterial members of the biofilm community, batch co-culture of single (or several) species of the other bacteria with *L. pneumophila*, may provide information on the interactions which occur between different species. It may be possible to determine which species are co-operative, which species are antagonistic, and which species are neutral to the growth of *L. pneumophila*.

This thesis has only been concerned with the ecology of *L. pneumophila*, the model and techniques used may have relevance to other waterborne pathogens. The microorganisms which could be studied in this type of system include bacteria (eg. coliforms) or parasites (eg. *Cryptosporidium parvum*).

CHAPTER 8
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MEDIA USED

TABLE 1 Reasoner and Geldritch medium

Ingredients (g/litre)	R2A	R3A
Yeast extract	0.5	1.0
Difco Proteose peptone no. 3	0.5	1.0
Caseamino acids	0.5	1.0
Glucose	0.5	1.0
Soluble starch	0.5	1.0
Sodium pyruvate	0.3	0.5
K ₂ HPO ₄	0.3	0.6
MgSO ₄ · 7H ₂ O	0.05	0.1
Agar	15.0	15.0
pH of the medium 7.2		

TABLE 2. Buffered charcoal yeast extract medium (BCYE)

Ingredients (g/litre)	
Yeast extract	10.0
Agar	12.0
Activated charcoal	1.5
<i>α</i> -ketoglutarate, monophosphate	1.0
ACES buffer	10.0
Potassium hydroxide	2.8
L-cysteine	0.4
Ferric pyrophosphate	0.25
pH of medium 6.9	

TABLE 3. GVPC medium
(Prepared as BCYE with the following additions)

Ingredients (g/liter)	
Glycine	3 g
Vancomycin	5 mg
Polymixin	11mg
Cycloheximide	80mg